Aus dem

Institut für Tierärztliche Nahrungsmittelkunde

Professur für Milchwissenschaften

der Justus-Liebig-Universität Gießen

Analysis of mycotoxins in the food chain by enzyme immunoassay: possibilities and limitations

KUMULATIVE DISSERTATION

zur Erlangung des Doktorgrades (Dr. rer. nat.)

im Fachbereich Agrarwissenschaften, Ökotrophologie und

Umweltmanagement der Justus-Liebig-Universität Gießen

vorgelegt von

Christina Rehagel

aus Frankfurt am Main

Gießen, August 2022

Mit Genehmigung des Fachbereichs Agrarwissenschaften, Ökotrophologie und Umweltmanagement der
Justus-Liebig-Universität Gießen

1. Gutachter:	Prof. Dr. Dr. habil. Ewald Usleber
2. Gutachterin:	Prof. Dr. Gesine Lühken
1. Prüferin:	Prof. Dr. Sylvia Schnell
2. Prüfer:	Prof. Dr. Mathias Fasshauer
Prüfungsvorsitzender:	Prof. Dr. Joachim Aurbacher
Tag der Disputation:	06. Dezember 2022

Meinen Eltern

List of publications included in this thesis

This thesis is based on three publications in peer-reviewed journals.

1. Rehagel, C., Akineden, Ö., Geisen, R., Cramer, B., Plötz, M., Usleber, E. (2022). Development and application of a competitive enzyme immunoassay for the detection of penitrem A in fungal mycelium: Evidence for frequent occurrence of multiple indole-containing mycotoxins in mouldy foods. *Food Control*, 108558. https://doi.org/10.1016/j.foodcont.2021.108558. (Impact Factor* 6.652)

2. Rehagel, C., Akineden, Ö., Usleber, E. (2022). Microbiological and mycotoxicological assessment of processed cereal-based complementary foods for infants and young children from the German market. *Journal of Food Science, 87,* 1810-1822. https://doi.org/10.1111/1750-3841.16106. (Impact Factor* 3.693)

3. Rehagel, C., Maul, R., Gützkow, K.L., Akineden, Ö. (2022). Enzyme immunoassays for the detection of mycotoxins in plant-based milk alternatives: pitfalls and limitations. *Mycotoxin Research*, *38*, 265-274. https://doi.org/10.1007/s12550-022-00467-x (Impact Factor* 4.082)

*Clarivate Journal Citation Reports, Science Citation Index Expanded, Journal Impact Factor 2021

Table of contents

List of abbreviations

AOH	alternariol
DON	deoxynivalenol
EC	European Commission
EIA	enzyme immunoassay
EFSA	European Food Safety Authority
GOx	glucose oxidase
Hbl	haemolytic enterotoxin
ICMSF	International Commission for Microbiological Specifications for Foods
IDTs	indole diterpene alkaloids
KLH	keyhole limpet hemocyanin
MEA	malt extract agar
ML	maximum level
Nhe	non-haemolytic enterotoxin
PAX	paxilline
PCF	processed cereal-based foods for infants and young children
PTA	penitrem A
SA	Sabouraud glucose chloramphenicol selective agar
T-2/HT-2	T-2/HT-2 toxin
ZEN	zearalenone

1 Introduction and objectives

Microbial contamination of plant-based food may occur at any stage of the food production process, including pre-harvest, harvesting, transport, storage, and processing, presenting a risk to human health (Los et al., 2018). While pathogenic bacteria are a threat mainly in raw plant materials, fungal toxins (mycotoxins) are not eliminated by heat treatment and other technologies used during food processing. Therefore, virtually all agricultural products of plant origin, raw materials and processed foods alike, are prone to a contamination with mycotoxins (Bullerman & Bianchini, 2007; Karlovsky et al., 2016). The main mycotoxigenic fungal species, belonging to the genera *Fusarium, Penicillium, Aspergillus, Alternaria,* and *Claviceps* are known to infect cereals (Pitt & Hocking, 2009). Therefore, mycotoxins are frequently detected in cereal products (Gottschalk et al., 2007, 2009), but also in animal feeding stuff (Penagos-Tabares et al., 2021; Santos Pereira et al., 2019; Vandicke et al., 2021). Although carry-over of mycotoxins from animal feed into food of animal origin has been described for a few compounds, such as aflatoxin M_1 in milk and ochratoxin A (OTA) in some pork tissues (Adegbeye et al., 2020), the main problem related to mycotoxins in food is plant-based foods.

The most important mycotoxins with regard to food safety are aflatoxins, OTA, fumonisins, patulin, zearalenone (ZEN), and trichothecenes (Alshannaq & Yu, 2017). Moreover, mycotoxins of the group of indole alkaloids, including ergot alkaloids and some indole diterpene alkaloids (IDTs) such as penitrem A (PTA), paxilline (PAX), lolitrem B, and epoxy-janthitrem may also occur in plant-based food (Andersen & Frisvad, 2004; Finch et al. 2013; Kalinina et al., 2018). The ecological requirements of mycotoxigenic fungi for survival and growth determine the degree of the risk of contamination of food commodities. Therefore, not all known mycotoxins are necessarily a risk in all plant-based foods, but different ingredients may contribute different toxins in composed foods.

In contrast to the highly regulated areas of primary agricultural production and professional food production, another potentially important source of mycotoxin exposure is relatively less studied, namely fungal spoilage of food in the consumer's household. Several mycotoxigenic fungal species are frequently found in this habitat, and even a minor colonisation with such fungi may render the products unsafe after a relatively short storage time.

With regard to bacterial contamination, including foodborne pathogens, the most vulnerable group of consumers are infants and young children. Starting at an age of about 4-6 months, plant-based material is gradually introduced in the diet of infants. Such products are not sterile, and may therefore contain pathogenic or toxigenic bacteria such as *Cronobacter sakazakii* and *Bacillus cereus* (Townsend & Forsythe, 2008). A possible co-occurrence of such pathogenic bacteria together with mycotoxins, which may or may not have an impact on health, unfortunately was hardly ever been studied so far. For this reason, microbiological risks were also considered in this work.

Although most major mycotoxins are regulated within the European Union and in many countries worldwide, and although routine control of main commodities for these mycotoxins is well established, there are still several “white spots”, lacking sufficient knowledge with regard to mycotoxin exposure of the consumer. Overall, there is a need for more information on the presence of non-regulated mycotoxins, especially IDTs, in non-standard food environments, in order to identify potential sources of intake and related risks for human health. These deficits of knowledge defined the objectives of this thesis. First, the risk from tremorgenic fungi isolated from mouldy foods, post-purchase at the consumer’s site, was assessed using a major compound, PTA, as a marker toxin. A second aspect is plant-based processed cereal-based food for infants and young children (PCF), for which very little information has been published so far. Finally, new trends in human nutrition, for example represented by the growing market of plant-based milk alternatives (PBMAs), potentially create new challenges for analytical methods for mycotoxins. This work covers all three topics, with enzyme immunoassay (EIA), as a commonly used routine analytical technique, being the primary tool of analysis. Since new sample materials require considerable efforts of method validation, liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were used for comparison, in cooperation with several other research institutes. A major aim of this thesis was to critically assess benefits and limitations of using rapid testing for mycotoxins in routine food control, not for regulatory or legal purposes, but as a part of quality control used in all stages of the food chain.

To achieve these objectives, new approaches to detect PTA by EIA were developed and applied in **study 1**, along some other existing methods for neurotoxic and tremorgenic mycotoxins. Established EIA methods were used to study new fields of application, for which new sample

preparation strategies had to be modified or newly developed, described in **study 2** (PCF) and **study 3** (PBMAs).

The first publication included in this thesis deals with the development of a new test system for PTA, followed by analysis of PTA and other indole alkaloids, namely PAX and ergoline alkaloids (ergot and clavine alkaloids), in mycelium extracts of fungal culture material isolated from naturally infected food materials. During this study, PTA production by a *Penicillium* species (*P. polonicum*) was reported for the first time.

The second study deals with the mycotoxicological and microbiological quality of PCF from the German market. Most food relevant mycotoxins together with microbiological investigations for hygiene parameters and relevant pathogenic bacteria were analysed as part of an application study.

The third study focuses on the application of different mycotoxin EIAs as rapid screening methods for direct testing of plant-based dairy alternatives to contribute to food safety of the products. A major outcome of this study was a description of the pitfalls and limitations related to test ruggedness towards sample matrix.

An overview of the analytical parameters which were covered by this thesis and some major properties of these target analytes is shown in **Table 1** (mycotoxins) and **Table 2** (pathogenic/toxigenic bacteria).

Table 1. Overview of mycotoxins covered in this thesis (**study 1-3**) and some important characteristics of these toxins.

Mycotoxin	Producers (Fungal genera)	Typical occurrence in	Adverse effects in mammals (human and/or animal)	Reference
AFB_1	*Aspergillus*	Cereals, peanuts, oilseeds	Genotoxic, carcinogenic, immunosuppressive, mutagenic, teratogenic	Benkerroum, 2020; IARC, 2012; Kumar et al., 2021
STC	*Aspergillus*	Cereals, soy beans, cheese, peanuts	Possibly carcinogenic, liver toxicity	Mol et al., 2016; IARC, 1987; Scott et al. 1972;
OTA	*Aspergillus, Penicillium*	Cereals, wine, nuts, cocoa, coffee, cheese, pork meat	Possibly genotoxic and carcinogenic, nephrotoxic, immunosuppressive, teratogenic	EFSA, 2006; IARC, 1993; Malir et al., 2013
PTA	*Penicillium*	Nuts, cheese, fruits	Vomiting, nausea, severe tremor, bloody diarrhoea brain damage, death	Botha et al., 2019; Cole et al., 1983; Gordon et al., 1993; Kalinina et al., 2018; Lewis et al., 2005
PAX	*Penicillium, Emericella, Claviceps, Epichloë*	Tomatoes	Sustained tremors and neurological disorders in avian and mammalian species	Andersen & Frisvad, 2004; Cole et al., 1974; Smith et al., 1997
Ergot alkaloids	*Claviceps, Aspergillus*, *Penicillium*, *Epichloë*	Cereals, cereal products	Disorders of the cardiovascular, nervous, reproductive, and immune systems	Coyle & Panaccione, 2005; Malysheva et al., 2014
DON	*Fusarium*	Cereals, cereal products	Vomiting, nausea, abdominal pain, feed refusal, immunosuppressive	Luo, 1994; Tanaka et al., 2002
T-2/HT-2	*Fusarium*	Cereals, cereal products, oats	Cytotoxic, skin toxicity, immunodepressive, haemorrhagic	Edwards, 2009 a, b, c; Gottschalk et al., 2007, 2009; Lutsky et al., 1978
ZEN	*Fusarium*	Cereals, cereal products	Hyperestrogenic syndrome	Kuiper-Goodman et al., 1987; Poor et al., 2015
AOH	*Alternaria*	Cereals, oilseeds, apples, tomatoes	Possibly cytotoxic, genotoxic, mutagenic, estrogenic effects	Ackermann et al., 2011; Aichinger et al., 2019; Brugger et al., 2006; Fehr et al., 2009; Scott, 2001

Table 2. Some important characteristics of foodborne pathogens analysed in **study 2**.

Bacterial pathogen	**Characteristics**	**Occurrence**	**Health effect**	**Reference**
(Presumptive) *Bacillus cereus*	Gram-positive, facultative-anaerobe, rod-shaped, endospore-forming	Ubiquitous, food and feed (vegetables, cereals, eggs, milk)	Food poisoning a) Diarrhetic toxins b) Emetic toxin (Cereulid)	Kramer & Gilbert, 1989; Stenfors Arnesen et al., 2008; Vilain et al., 2006
Cronobacter spp. (*C. sakazakii* and several other species, formerly named as *Enterobacter sakazakii*)	Gram-negative, facultative-anaerobe, non-sporeforming, motile, peritrichous rods	Plant materials, food industry environment (*e.g.* cereals, vegetables, water, vegetables, milk, food production sites [recontamination])	Meningitis, sepsis, death, necrotizing enterocolitis in infants and neonates	Baldwin et al., 2009; Bar-Oz et al. 2001; Mashoufi et al., 2017; Van Acker et al., 2001

2 Scientific background

2.1 Mycotoxins under study

Aflatoxin B_1 (AFB_1) is the dominant compound within the aflatoxins, carcinogenic mycotoxins produced by several species of *Aspergillus,* mainly by *A. flavus* and by *A. parasiticus*. AFB_1 plus three other aflatoxins (AFB_2, AFG_1, AFG_2) are considered to be most relevant in food and feed, therefore European Commission regulation (EC) No. 1881/2006 covers both AFB_1 individually, and the sum of the four toxins (EC, 2006). AFB_1 AFB_2, AFG_1, AFG_2, and the AFB_1 metabolite in milk, AFM_1, are all considered as "class 1" known human carcinogens (IARC, 2012). AFB_1 has already been detected in foods including maize, rice, oilseeds, dried fruits, legumes (soy, groundnuts) and nuts such as hazelnuts, pistachios, and brazil nuts (Kumar et al., 2021). The toxin is known to possess various biological activities, including acute toxicity, teratogenicity, mutagenicity, and carcinogenicity (Benkerroum, 2020).

The aflatoxin analogue **Sterigmatocystin** (STC) is an end product in *A. versicolor*, and a precursor of aflatoxin biosynthesis in *A. flavus* (EFSA, 2013). STC have been detected in food such as cereals and cereal products (Mol et al., 2016) and also in interior environments (Engelhart et al., 2002). In 1987, STC was classified as a "class 2B" carcinogen (possible human carcinogen; IARC, 1987) and is less toxic compared to AFB_1 (Wong et al., 1977).

OTA is produced by some species within the genus *Penicillium* and *Aspergillus*, most notably by *A. ochraceus* and *P. verrucosum*. The most notorious chronic effect of OTA exposure is kidney necrosis, but also has immunosuppressive effects, and possibly is genotoxic (Malir et al., 2013). OTA has been found in a large number of plant food materials worldwide, including cereals, grapes, coffee beans, and cocoa (EFSA, 2006). Some carry-over into pork products has been reported, although is of relatively minor importance compared with plant-based foods (EFSA, 2006). The IARC classified OTA as a possible human carcinogen (Group 2B) in 1993 (IARC, 1993), but recent data on OTA genotoxicity point towards the need for reclassification into group 1 (Stoev, 2022).

IDTs are a large and highly diverse group of mycotoxins, which are produced by a wide range of different fungi **(Table 1**). These compounds are divided into several subgroups based on their chemical structure, including paspalins, paxillins, shearinins, paspalitrems, terpendoles, penitrems, lolitrems, janthitrems, and sulpinines (Saikia et al., 2008; Reddy et al., 2019). IDTs

have a common backbone composed of a cyclic diterpene backbone, derived from geranylgeranyl diphosphate, and an indole component, derived from tryptophan (Acklin et al., 1977; De Jesus et al., 1983). In the further course of their biosynthetic pathway, enzymatic reactions take place at different sites of this backbone, resulting in the structural diversity of these compounds (Reddy et al., 2019).

IDTs block calcium-activated potassium channels in the nervous system, causing neurotoxic and tremorgenic effects in insects and mammals (Imlach et al., 2011; Knaus et al., 1994). There is still a lack of data on the potency of many naturally occurring IDTs, most information is available for herbivores because they are exposed to pastures dominated by endophyte-infested grasses in several countries (Cole et al., 1974; Smith et al., 1997; Mantle & Penn, 1989). With regard to toxicity of IDTs occurring in food, some data are available for PTA, which is mainly produced by *P. crustosum* (Eriksen et al., 2013). However, neither tolerable daily intake data nor maximum levels (MLs) in foods have been established for any IDT so far. PTA has been found in about 10% of cheeses from several European countries, with levels up to 429 µg/kg (Kalinina et al., 2018).

Ergoline alkaloids are a group of about 80 indole-containing mycotoxins sharing a common four-ring backbone and are produced by species from several different fungal genera, including *Claviceps*, *Epichloë, Aspergillus* and *Penicillium* (Panaccione, 2005). Ergoline alkaloids are divided into three groups according to their chemical structure: clavines, ergoamides, and ergopeptides (Chen et al., 2017). The main producer of ergot alkaloids is *C. purpurea*, which primarily produces seven ergot alkaloids (alpha- and beta-ergocryptine, ergocristine, ergotamine, ergosine, ergometrine, ergocornine) plus their respective epimeric forms (-inine). With exception of ergometrine, these ergot alkaloids belong to the subgroup of ergopeptides (Pierri et al., 1982). Ergot alkaloids may contaminate all cereal grains, notably rye, but also barley, wheat, millet, oats, and triticale (Malysheva et al., 2014). A major producer of clavine-type ergolines is *P. roqueforti*, most isolates from blue-veined cheese produce significant levels of isofumigaclavines (Scott, 1981). Due to their structural similarity to the neurotransmitters adrenaline, noradrenaline, dopamine, and serotonin, ergoline alkaloids exhibit strong interactions with the corresponding receptors in the central nervous system as well as in blood vessels, causing disorders in cardiovascular, nervous, reproductive, and immune system (Coyle & Panaccione, 2005).

The term ***Fusarium* toxins** comprise of many, structurally different mycotoxins, such as the trichothecenes, the fumonisins, and ZEN, Deoxynivalenol (DON; also named vomitoxin by Vesonder et al., 1973), is considered to be the most important trichothecene (Sobrova et al., 2010; Canady et al., 2001), commonly co-occurring together with the estrogenic ZEN in virtually all cereals worldwide. T-2 toxin, which normally occurs together with HT-2 toxin, is a major problem in oats. In mammals, T-2 is converted to HT-2 by the process of deacetylation during digestion of contaminated food or feed (Marin et al., 2013; Krska et al., 2014).The estrogen-like compound ZEN frequently co-occurs with trichothecens in all cereals, which fumonisins are most exclusively are a problem in maize (Miller & Trenholm, 1994). Mycotoxins belonging to trichothecenes are characterised by a tricyclic backbone with an epoxy group at C-12 and C-13, which is responsible for toxicity, and a double bond at C-9 and C-10 (Mirocha & Christensen, 1974). Trichothecenes bind to ribosomes, interfere with translation and cause apoptosis in proliferating cells (Rosenstein et al., 1979; Ueno, 1984; Jaradat, 2005). In addition, they activate intracellular protein kinases that mediate selective gene expression and apoptosis, resulting in pathological consequences (Pestka, 2008).

Mycotoxigenic fungi of the genus *Alternaria* are widespread in the environment and have been found in a variety of foods, especially in cereal-based foods (Ostry, 2008). Cereal contamination with **alternaria mycotoxins** may occur during growth in the field (Logrieco et al., 2009), but also during storage of raw cereals, as some species are capable to grow at low temperatures (Ozcelik et al., 1990). To date, at least 70 compounds with toxic effects have been isolated from *Alternaria* fungi, including alternariol (AOH; Arcella et al., 2016). Based on its chemical structure, AOH belongs to the group of dibenzo-α-pyrones (King & Schade, 1984). *In vitro* studies on the toxicity of AOH have shown cytotoxic, genotoxic, mutagenic and estrogenic effects (Aichinger et al., 2019; Brugger et al., 2006; Fehr et al., 2009).

2.2 Regulations for mycotoxins in food

The toxicity of mycotoxins has led many countries to establish MLs for mycotoxins in food and feed in order to minimise the health risk for consumers and animals. The European legislation has set MLs for mycotoxins in foodstuffs in the Commission Regulation (EC) No. 1881/2006 of 19 December 2006. This regulation contains MLs for the following mycotoxins: Aflatoxins (B_1, B_2, G_1, G_2, M_1) (AFB_1 in PCF: 0.1 µg/kg) fumonisins (B_1, B_2), OTA (OTA in PCF: 0.5 µg/kg), ZEN

(ZEN in PCF: 20 µg/kg), DON (DON in PCF: 200 µg/kg), Patulin, and Citrinin. With regard to ergot alkaloids, the European Union has published new MLs for ergot sclerotia and ergot alkaloids in certain foodstuffs in Regulation (EU) 2021/1399 amending Regulation (EC) No. 1881/2006, including PCF with an ML for ergot alkaloids of 20 µg/kg. In relation to T-2/HT-2, there is currently no ML, but the European Commission (EC, 2013) recommended indicative levels for the sum of T-2/HT-2 for cereals and cereal-derived products (T-2/HT-2 in PCF: 15 µg/kg). In the case of *Alternaria* toxins, the European Commission has recently published indicative levels and a monitoring recommendation for certain food categories, including PCF with an indicative level for AOH of 2 µg/kg (EC, 2022). Regarding the ML for IDTs, there are no regulations for penitrems or any other IDT in foods and feeds, and therefore IDTs are not usually included in routine mycotoxin control of foods.

2.3 Immunoassays as a routine tool in mycotoxin analysis

Analytical methods for the detection of mycotoxins in food and feed include a variety of techniques, with chromatographic methods being primarily used (Janik et al., 2021). These methods include thin layer chromatography (TLC), high performance liquid chromatography (HPLC) in combination with different detection methods (diode array, fluorescence, UV detection), LC-MS/MS and gas chromatography-tandem mass spectrometry (GC-MS/MS) (Janik et al., 2021). However, these methods are relatively laborious due to necessary sample preparation methods and require expensive instrumentation equipment. In contrast, immunochemical methods offer a cost-effective and rapid method for the detection of mycotoxins. EIAs, fluorometric assay with immune affinity column (IAC) clean-up or fluorometric assay with solid phase extraction (SPE) clean-up are used for quantitative detection of mycotoxins, while flow-through immunoassay and lateral flow test are used for semi-quantitative determination of these compounds (Zheng et al., 2006).

IDTs in particular are currently hardly considered in the routine diagnostics of mycotoxins, which is probably also due to the lack of easy and fast detection systems. Currently, various physicochemical methods are used for the detection of, for example, PTA in food and other sample materials, in particular LC-MS/MS, in addition to TLC- and GC- methods (Tor et al., 2006). For many mycotoxins, EIAs or other immunochemical methods have already been described, and for the toxins with MLs (e.g., aflatoxins), a variety of commercial immunoassays

are available (Anfossi et al., 2013; He et al., 2018). For PTA, however, no such test system has yet been described, even the production of specific antibodies has not yet been described in the literature.

2.4 Pathogenic and toxigenic bacteria in PCF

Cronobacter spp. belongs to the family of *Enterobacteriaceae*. Seven *Cronobacter* species have been identified to date (*C. sakazakii, C. malonaticus, C. turicensis, C. condiment, C. muytjensii, C. dubliniensis, C. universalis)* (Iversen et al., 2008), and have been associated with invasive infections such as necrotizing enterocolitis, meningitis and septicemia, particularly in infants and neonates through consumption of powdered infant formula. Infections in infants are only associated with strains of *C. sakazakii, C. malonaticus* and *C. turicensis* (Kucerova et al., 2010), with *C. sakazakii* being the most frequently isolated (Müller et al., 2013). *C. sakazakii* is an opportunistic foodborne pathogen, which shows strong resistance to extreme environmental conditions such as desiccation, heat, acid and osmotic stress (Fakruddin et al., 2014). Because of these properties, *C. sakazakii* is found not only in food production environments but also in clinical environments (Mashoufi et al., 2017). Since *Cronobacter* has already led to several deaths, it was classified as “severe hazard for restricted populations, causing life threatening or substantial chronic sequelae or illness of long duration” by the International Commission for Microbiological Specifications for Foods (ICMSF) in 2002 (ICMSF, 2011).

The ***B. cereus*** group consists of several genetically closely related species that are widely distributed in the environment and has therefore already been detected in many types of food of plant and animal origin (Kramer & Gilbert, 1989). Species within this group are difficult to distinguish from each other by cultural and biochemical methods and are therefore called "presumptive *B. cereus*". Members of the *B. cereus* group are able to form endospores that are highly resistant to extreme conditions of UV-rays, heat, pH, and desiccation, making it difficult to inactivate or remove them from the food chain (Messelhäußer & Ehling-Schulz, 2018). *B. cereus* is capable of causing food poisoning in two different forms named emetic and diarrheal syndrome. The emetic syndrome is caused by the heat-stable toxin known as cereulide, which is produced by *B. cereus* in the food matrix prior to ingestion. In diarrhoeal syndrome, toxin-producing *B. cereus* strains are ingested with food and subsequently form

protein-toxin complexes in the intestine, such as non-haemolytic enterotoxin (Nhe) and haemolytic enterotoxin (Hbl), as well as single proteins such as cytotoxin K (Ehling-Schulz et al., 2004; Messelhäußer & Ehling-Schulz, 2018).

3 Brief summary of the three studies included in this thesis

3.1 Study 1:"Development and application of a competitive enzyme immunoassay for the detection of penitrem A in fungal mycelium: Evidence for frequent occurrence of multiple indole-containing mycotoxins in mouldy foods"

3.1.1 Objectives

Based on previous studies, PTA has already been detected in various contaminated foodstuff such as nuts (Botha et al., 2019), cream cheese (Young et al., 2003), soup (Lewis et al., 2005) beer (Cole et al., 1983) and food wastes (Rundberget et al., 2004). In some cases, consumption of contaminated food has led to mycotoxicosis in animals or humans, but a clear causal relationship between PTA intake and clinical symptoms could not be established in any of these cases. Therefore, the objective of this study was to collect more data on the occurrence of this mycotoxin in addition to other indole alkaloids to identify potential sources of intake and associated toxic risks to humans. To this end, anti-PTA antibodies were generated, which were subsequently used to develop a competitive EIA. Since production of antibodies against PTA has not been reported so far, strategies were established to prepare polyclonal anti-PTA antiserum and PTA-labeled solid phase antigens for use in competitive EIA. For this purpose, PTA was coupled to keyhole limpet hemocyanin (KLH) and glucose oxidase (GOx), using the Mannich condensation-reaction. Rabbits were immunised with the immunogen PTA-KLH. The PTA-EIA, together with two EIAs for PAX and ergoline alkaloids, were used to study the toxin profiles in culture of fungi isolated from some naturally infected food sample materials, including dairy products, citrus fruits, and walnuts. Finally, the plausibility of the EIA results was evaluated by comparison with LC-MS/MS, and by characterisation of some of the PTA-producing fungi.

3.1.2 Results

In this study, novel polyclonal antibodies against PTA were produced and were used to develop an indirect competitive EIA. Therefore, the Mannich condensation reaction using formaldehyde (Mannich & Krösche, 1912) was used to couple PTA to carrier proteins KLH and GOx. The PTA-KLH conjugate was used for the immunisation of rabbits, whereas the PTA-GOx conjugate was used as solid phase antigen. In indirect EIA, PTA specific antibody titers could

be detected in three out of four rabbits with maximum titers of about 1:10,000. The standard curve enabled detection of PTA in the ng/mL range and had a background absorbance of about 45% B/B_0. The mean (n=7) PTA standard curve parameters IC_{50} and IC_{20} were at 3.1 ± 1.4 µg/mL and 0.22 ± 0.17 µg/mL, the latter set as the detection limit. The test system showed no competitive binding inhibition by other IDTs such as PAX, verruculogen, terpendole E, and lolitrem B, each at a level of 1 µg/mL.

For cultivation of fungal cultures, fungal material from contaminated foodstuffs (dairy products, citrus fruits, walnuts) was transferred to malt extract agar (MEA) and Sabouraud glucose chloramphenicol selective agar (SA). According to colony morphology, most moulds could be identified as *Penicillium* species. While fungal material obtained from each sample of cheese and citrus fruit had uniform macroscopic appearance, fungal material from walnuts (fruit or shell) consistently grew as mixed cultures, and both *Penicillium* and *Aspergillus* type structures were found to be dominant according to microscopic evaluation.

Mycelium extracts were then prepared in methanol by cutting out 1 cm² of agar and adding to 1 ml of methanol. The mixture was then centrifuged, the supernatant was collected and analysed in EIA. Analyses of mycelium extracts of fungal cultures from MEA and SA by the three EIAs yielded positive results for 19 out of 27 isolates in at least one test system. PTA was produced by eight out of 27 mycelium extracts from MEA and SA (2-200 µg/mL). Ten isolates from all types of food were also positive in the PAX-EIA (0.004-4 µg/mL). The EIA for ergoline alkaloids yielded positive results (0.0002-0.4 µg/mL) in isolates from dairy products and from walnuts, but not from citrus fruits. HPLC-MS/MS was used as a control method for PTA and PAX in four selected mycelium extracts (N4; N11; N19; N25) and could qualitatively confirm the EIA results, but resulting in poor quantitative agreement.

In order to further characterise the results obtained by the EIAs, the three isolates for which the highest PTA levels had been found in mycelium extracts (N4; N11; N19), had been selected for further identification. Sequencing of the ITS region and partial sequencing of the β-tubulin gene (Visagie et al., 2014) clearly identified isolate N4 (from cheese with herbs) as *P. polonicum*, whereas isolates N11 (citrus) and N19 (walnut) were both identified as *P. crustosum*. This was in agreement with colony morphology on MG and MA.

3.1.3 Discussion

The result of **study 1** was that a novel EIA for the detection of PTA was developed, which enabled a moderately sensitive detection of this toxin. The standard curve allowed detection of PTA in the ng/mL range (LOD: 220 ng/mL) but had a relatively high background absorbance of about 45% B/B_0. Thus, the PTA-EIA had a much higher detection limit compared to previous studies of, for instance, Kalinina et al. (2018) (LOD: 0.36 ng/mL) or Rundberget and Wilkins (2004) (LOD: 5 ng/mL). The application of this PTA-EIA alongside established EIAs for the detection of PAX (LOD: 0.7 ng/mL; Bauer et al., 2018) and ergoline alkaloids (LOD: 0.02 ng/mL; Gross et al., 2018) in mycelium extracts of fungal cultures isolated from various foods demonstrated that these indole alkaloids are frequently occur in mouldy foods. Another remarkable finding of **study 1** was that for the first time, *P. polonicum* could be identified as a PTA-producer.

In terms of test sensitivity, the PTA-EIA, with a mean detection limit of the standard curve of 220 ng/mL, was not sensitive enough to analyse PTA in dilute extracts of food samples at levels that occur under natural conditions. For this reason, the first application of the test system was the study of PTA producing capacity of fungi obtained from slightly mouldy food such as dairy products, citrus fruits, and walnuts.

With the exception of PTA, no analytical toxin standards were available for the other penitrems B to F, so cross-reactivity of these compounds could not be determined in the PTA-EIA, which was a major shortcoming of the study. Instead, structurally related IDTs such as PAX, verruculogen, terpendole E and lolitrem B were tested. The PTA-EIA showed no cross-reactions with these substances, which was not unexpected because of the major structural differences between these compounds and PTA.

To verify the reliability of the test system, selected mycelium extracts were analysed for PTA and PAX by HPLC-MS/MS. Based on the fact that qualitative toxin standards of penitrems B to F were available from the study by Kalinina et al. (2018), these substances could be detected by HPLC-MS in all extracts studied. It was assumed that the total content of penitrems was dominated by substances other than PTA. This indicated that the PTA-EIA also covers analogues, suggesting its potential for group specificity. In this case, this can be interpreted as an advantage of EIAs if they could indicate the presence of toxins that cannot yet be

quantified. This may also explain why previous studies have failed to assign plausible mycotoxin levels to poisoning cases (Botha et al., 2019; Richard et al., 1981).

The HPLC-MS/MS analyses for PAX revealed a signal in two out of four mycelium extracts examined. Furthermore, a signal with the characteristic m/z value and fragmentation pattern of 13-desoxy-PAX could be detected in all mycelium extracts that were positive in the PAX-EIA, indicating that the PAX-EIA most likely cross-reacts with this derivative to a considerable extent as previously observed by Bauer et al. (2018). Based on these findings by HPLC-MS/MS, the poor quantitative agreement between the two methods could also be explained.

Penicillium spp. capable to produce PTA, PAX, and ergoline alkaloids, either alone or in combination, appear to be quite common as contaminants on foods at the retail or production level. Some are capable to produce PTA in culture at high levels, which would have to be considered as toxic if present in food. In comparison, Kalinina et al. (2018) detected maximum concentrations of PTA of 429 µg/kg directly in cheese from the German market. These observations show that further studies on the presence of IDTs in the food chain are required, including the development of rapid detection methods to assess the risk to humans by the presence of PTA and other IDTs in food.

In addition to the field of food hygiene, the application of an optimised PTA-EIA is conceivable, for example, in the analysis of cases of poisoning in dogs described in the literature, as well as for the investigation of feedstuffs based on the reports of PTA in silage (Gordon et al., 1993) and could thus also be used in the field of animal health.

3.2 Study 2:"Microbiological and mycotoxicological assessment of processed cereal-based complementary foods for infants and young children from the German market"

3.2.1 Objectives

Due to the increasing demand for organically produced food, food safety studies of organically produced PCF products are required, especially due to the fact that organic farming systems allow only restricted use of pesticides. Previous studies on microbial contamination in PCF have shown that these products may be a potential source of mycotoxins (Al-Taher et al., 2017; Assunção et al., 2018; Braun et al., 2020; Gotthardt et al., 2019; Juan et al., 2014; Oueslati et al., 2018; Pereira et al., 2015) and bacterial pathogens (Kim et al., 2011; Ziver et al., 2020).Since very little information is available on the microbiological and mycotoxicological quality of organic PCF from the German market, the aim of this study was to provide a comprehensive overview of the occurrence of some mycotoxins, DON, ZEN, AOH, T-2/HT-2-toxin, and ergot alkaloids and the level of microbiological contamination of these products. Mycotoxin analyses were performed by competitive EIA methods using established extraction methods. Bacteriological quality of samples was checked by analysing for some relevant microbiological criteria, namely aerobic mesophilic bacteria, mould count, *Enterobacteriaceae*/*Acinetobacter, Cronobacter* spp., and presumptive *Bacillus cereus* according to ISO methods. Some samples showing suspect colonies were further identified by biochemical methods and 16S rDNA sequencing to obtain more information on the spectrum of the bacterial species in PCF.

3.2.2 Results

The result of **study 2** was the detection of low concentrations of DON, T-2/HT-2 (in all 15 oat-based products), and AOH in PCF from the German market by established EIAs. ZEN and ergot alkaloids could not be detected in any case. A further important finding was that a few samples contained low numbers of opportunistic pathogens, most notably *Cronobacter sakazakii*, *Acinetobacter* spp., *Pantoea* spp., and enterotoxigenic *Bacillus wiedmannii.*

The first part of the study examined PCF products from the German market on the occurrence of DON, T-2/HT-2, AOH, ZEN, and ergot alkaloids. To this end, PCF products were purchased from all major companies offering such products at local retail stores, drugstores, and organic specialty stores. Single-grain products (*n*=25) contained millet, spelt, oats, wheat, barley, rye,

rice, or maize. Multigrain products (*n*=13) contained at least two, up to seven, of these cereals. Extraction methods for all mycotoxins in PCF were performed according to earlier studies (Liesener et al., 2010), except for AOH which was extracted according to the method of Ackermann et al. (2011). All procedures had to be adopted to meet the requirements of the highly absorptive PCF matrix. For each mycotoxin EIA, the mean LOD as indicated by Liesener et al. (2010) was checked and verified, using spiked PCF sample material. The recovery experiments showed that the mean recovery rates for DON, T-2/HT-2, and AOH in spiked samples were in the range of 69%–120%. For ergot alkaloids and ZEN, which were also analysed in all samples, higher recoveries ranging from 115% to 160% were determined. Further, three samples containing each of the highest toxin level of DON, T-2/HT-2, and AOH were spiked with the respective toxin and reanalysed. After subtracting the measured toxin content of the nonspiked sample, recoveries of 77%–106% were obtained. Myxotoxin analyses by EIAs revealed that ergot alkaloids and ZEN were not detectable in any sample. DON was found in 10 samples (9-35 µg/kg). A considerable part of the samples (66 %) was positive for AOH (0.4-2 µg/kg). All samples containing oats (*n*=15) were positive for T-2/HT-2 toxin (1-8 µg/kg).

The second part of the study examined the microbiological quality of the PCF products. With regard to the microbiological results, all products analysed showed low or very low contamination. Most samples had very low aerobic mesophilic cell counts ($<2.0 \times 10^1$ CFU/g), the maximum was 9.6×10^2 CFU/g. A few samples contained low numbers of opportunistic pathogens, most notably *Cronobacter sakazakii*, *Acinetobacter* spp., *Pantoea* spp., and enterotoxigenic *Bacillus wiedmannii*.

3.2.3 Discussion

Study 2 was the first analysis of the PCFs from the German market, and the second one worldwide (Assunção et al., 2021), in which two major food safety categories have been studied, namely microbiological quality and contamination with mycotoxins.

Within the recovery studies, high values were found for ZEN and ergot alkaloids. The high recoveries obtained for ZEN may be explained by some remaining matrix interference in the three selected materials, because the blank materials used for spiking were clearly below the LOD but yielded absorbance values of 93%–98% B/B0 × 100. Likewise, the high recovery rates

for ergot alkaloids may also be explained by some remaining matrix effects. Since both tests yielded toxin negative results for all samples, no attempt was made to further improve sample extract preparation. Further studies will aim at optimizing the overall analytical strategy, including sample extraction and confirmation of positive results by, for example, an LC-MS/MS reference method as used in **study 1 and 3**. Moreover, three samples containing the highest toxin content of DON, T-2/HT-2 and AOH were spiked with the respective toxin content determined by EIA and reanalysed. The recoveries determined in this process (77%-106%) demonstrated a toxin-dependent additive effect and further indicates the suitability of the EIA methods.

While the sample extraction methods and overall method performance were considered to be sufficient, the relative cross-reactivities of the EIAs potentially affected the accuracy of the toxin content determined by EIA as in **study 1** in the case of PTA, PAX and ergoline alkaloids. Consequently, the true toxin content for T-2/HT-2 and ergot alkaloids might be underestimated, whereas the results for DON and AOH might be overestimated due to reactivity with toxin analogues.

While for all mycotoxins analysed, the LODs were well below the established MLs, the LOD of the EIA method for ergot alkaloids (30 µg/kg) did not fully meet the recently published European Union ML for this group of toxins in PCF (20 µg/kg), but still are at a very similar level.

All samples analysed yielded clearly ergot alkaloids- and ZEN-negative results. The complete absence of detectable levels of ZEN and ergot alkaloids may be explainable by careful cereal cleaning and selection and procedures. DON was found with relatively high frequency (26%) but at low levels (maximum concentration: 35 µg/kg) in PCF products, regardless of cereal composition, which may be explained by not fully efficient removal during cereal processing (Karlovsky et al., 2016). Similar findings have been reported by others (Herrera et al., 2019; Juan et al., 2014; Pereira et al., 2015). T-2/HT-2 were found in all 15 oat-containing products, but again at very low concentrations (1-8 µg/kg). Our results are consistent with the findings of Al-Taher et al. (2017), who reported low levels of T-2/HT-2 (<10 µg/kg) in oat-based and mixed-grain infant cereals from the U.S. market. It was not surprising that a considerable part of the samples (66%) from each of the six product categories was positive for AOH (0.4–2 µg/kg), because AOH is the most frequent occurring *Alternaria* mycotoxin in food (EFSA, 2011).

Similar levels of AOH (<10 µg/kg) in single grain as well as in multi-grain baby foods had also been reported by Scott et al. (2012) and Gotthardt et al. (2019). In addition, co-occurrence of trichothecenes and AOH was detected in some samples, consistent with results previously reported for PCF products (Juan et al., 2014; Zhang et al., 2018).

3.3 Study 3:"Enzyme immunoassays for the detection of mycotoxins in plant-based milk alternatives: pitfalls and limitations"

3.3.1 Objectives

According to studies on the occurrence of mycotoxins in PBMAs, PBMAs are known to be a potential source of mycotoxins of different groups (Arroyo-Manzanares et al., 2019; Hamed et al., 2017; Hamed et al., 2019; Juan et al., 2022; Miró-Abella et al., 2017). For liquid food materials, EIAs appear to be a suitable tool for rapid on-site testing and have been used for decades for the analysis of aflatoxin M_1 in cow's milk (Pecorelli et al., 2020). Consequently, **study 3** examines the hypothesis of the suitability of the EIA as a rapid screening tool for direct analysing of PBMAs without sample extraction. For this purpose, first the matrix effect of different PBMA matrices (soy, almond, oat, mix-based) on competitive EIAs for the detection of AFB_1, STC, OTA, DON, and T-2/HT-2 was tested. Mycotoxin analysis using these EIAs was then performed on samples from the German market. In this study, the LC-MS/MS method was used again to confirm highly positive EIA samples regarding AFB_1, STC, and OTA.

3.3.2 Results

The result of **study 3** was that the use of mycotoxin EIAs for direct testing of PBMAs without proper sample preparation can lead to false-positive or false-negative results due to matrix interferences caused by ingredients of PBMAs.

Initially, various PBMA products were purchased in retail stores and special "health food stores". The main ingredients were water as well as various vegetable substances (cereals, legumes, nuts, seeds, pseudo cereals). Some products contained small amounts of sunflower or rapeseed oil, cocoa, sugar and salt.

At the beginning of the study, the extent of the matrix effect was investigated. However, the problem at this point was that no negative reference materials were available, so all PBMA products were first analysed at different dilutions with buffer solution. Then, selected samples of each product group were used to establish toxin standard curves in matrix. It was found that PBMA matrix affected each individual EIA test system to a different extent. Consequently, a minimum dilution factor of 8 was obtained for all EIAs except STC with a minimum dilution factor of 20.

Within the recovery studies, values for all mycotoxins in major product groups ranged from 56-156%. After analysis of PBMA products from the German market, positive results in at least one test system were obtained for 43% of samples. However, most positive results were near the calculated detection limit. A concomitant LC-MS/MS for analysis of highly positive samples and their extracts (IAC/LLP) for AFB_1, STC and OTA was performed. EIA results showed that LLP extracts were still positive, albeit at lower levels, in the tests for AFB_1 and STC. IAC extracts were all EIA negative for AFB_1, but still weakly positive for STC. However, LC-MS/MS could qualitatively confirmed the presence of trace amounts of STC in some samples, but quantitative agreement was poor.

3.3.3 Discussion

The necessity to dilute PBMAs for EIA analyses negatively affected the achievable, calculated detection limit in sample matrix. The calculated LODs in PBMAs were considered to be still in a relevant concentration range for DON (LOD: 16 µg/L) and T-2/HT-2 (0.4 µg/L) while for AFB_1 (0.4 µg/L), STC (2 µg/L), and OTA (0.08 µg/L) they were probably insufficient.

Recovery studies also reflected the extent of the PBMA matrix, yielding results which were still quite variable (56-156%), depending on both, type of matrix and spiking level. This indicates that even at a minimal dilution factor of 8 (STC-EIA: 20), some remaining matrix interference could cause up to 50% deviation from the nominal value. A possible reason for these matrix interferences are the proteins contained in PBMAs. Matrix interferences were also observed in recovery studies in **study 2** for EIAs for ZEN and ergot alkaloids, albeit at lower extent.

In **study 3**, the EIAs for DON and T-2/HT-2 yielded plausible results based on trichothecene frequency in cereals, and agreed well with data on contamination of oats (Curtui et al., 2009; EFSA, 2017) and cereals in general (Gottschalk et al., 2009). In the remaining EIAs for AFB_1, STC, and OTA, some oat-based, soy-based, or pea-based samples containing cacao, in particular, yielded conspicuous results in addition to one sample based on black whole grain rice. However, the AFB_1-EIA gave negative results for almond-based PBMAs, which was rather atypical as almonds are known to potentially contain aflatoxins (Kanik & Kabak, 2019). The

reason for these results could be that the LOD for this toxin does not allow a sufficiently sensitive analysis in these matrices.

Further work on elucidation of matrix effects therefore focussed on products containing cocoa as an ingredient and the product based on black whole grain rice. In fact, these products yielded the highest results in the AFB_1-EIA, STC-EIA or OTA-EIA. In an initial attempt to improve the detection limit by lowering the sample dilution factor, extracts were prepared by LLP of these samples with ethyl acetate, followed by a further clean-up step on IAC columns. The results of this comparison analysis showed virtually no agreement between EIA and LC-MS/MS. Furthermore, the EIA results for diluted sample and sample extracted by LLP or IAC also gave fully inconsistent results. This indicates that at least for cocoa-containing samples, the EIAs are not applicable to PBMA without significant improvement of the sample preparation method. Further work will study on a broader sample matrix basis, whether similar discrepancies are to be expected for other PBMA products.

4 Synopsis: major outcomes and new questions

The primary motivation of this thesis was to contribute to the improvement of food safety. Without suitable methods to detect possible health risks, food safety cannot be ensured. Although mass spectrometric methods are nowadays the preferred methods of analysis, the costs of analysis, and the requirement of highly skilled personal will for the foreseeable future limit the use of such methods to a laboratory environment. If rapid results are required at reasonable costs, EIA still play an important role in food control, most notably for point-of-care quality control in the food industry. However, the basic principles of EIA require that for each and every field of application, control analyses have to be performed in order to avoid pitfalls related to sample matrix composition, pH, fat content, enzyme inhibitors, just to name a few. However, the potential of EIA is not just restricted to rapid testing. For example, search analyses of new sample matrices for a certain contaminant can be performed without much laboratory efforts needed, provided that any positive result is subjected to a critical scrutiny. Another feature of immunoassay, namely cross-reactivity with structurally related compounds, may be helpful within such search analysis. It is important to note that for natural contaminants such as mycotoxins, the target toxin of an EIA rarely exists as a single compound in a sample, but analogues, precursors, and modified forms may also be present (Rychlik et al., 2014). The EIA for PTA which was developed in **study 1** was the first one antibody-test for this toxin. Although it is known that many PTA analogues exist, cross-reactivity could not be determined because no standards are available except of PTA. However, when applied to mycelium extract of several mould isolates, PTA positive extracts could easily be identified for further analysis by LC-MS/MS. Using this strategy, production of the full spectrum of penitrems A-F was detected in all positive samples. This indicates that the numerical overestimate of PTA by EIA compared with LC-MS/MS is most likely the result of cross-reactivity of the other penitrems in this assay. Furthermore, a new PTA-producing fungal species, *P. polonicum*, isolated from cheese with herbs, could be identified by using the PTA-EIA for pre-screening. Overall, the ease of use and the possibility to rapidly change the test procedure of the microtiter plate format according to analytical requirements is certainly an advantage over other methods of analysis.

In the second study, another advantage of EIA could be demonstrated. If the vast majority of a given number of samples is negative for the target toxin, the sample throughput of EIAs can

be very high. PCF were chosen as an example because very few published data exist for this product category, and because infants are the most vulnerable group of consumers. Fortunately, all analytical results for all groups of toxins were well below critical levels. The overall number of positive results, and the levels of mycotoxins in positive samples, were very low in all products of PCF. Because the recovery and repeatability of the test systems was extensively studied, the likelihood of false-negative results appeared to be minimal. One hypothesis of the study was that the overall level of good manufacturing practice will not be restricted to a single group of parameters (mycotoxins), but will also include other food safety criteria. In fact it was shown that not just mycotoxin levels in the studied PCF were satisfying, but also the microbiological quality of these products.

PBMAs were selected in **study 3** because there is an emerging market for this type of foods, and because little is known with regard to mycotoxin contamination of such products. Considering the large variety of composition of products on the German market, it was of importance not to treat the different products (oats, soy, almond, plus some others) as a uniform matrix. It was shown that, although liquid sample material is well suited for analysis by EIA, simple dilution could not overcome strong and varying matrix interference in some assays, most notably the tests for AFB_1 and for STC. Although the degree of matrix interference was surprisingly high in some tests, others were much less affects, for example the tests for trichothecenes. Although this study mostly demonstrated the risk of pitfalls of EIAs, some results strongly suggest that further efforts are necessary, because these PBMAs may contain mycotoxins at levels which have to be taken seriously. It is also important to develop a suitable means for sensitive detection of mycotoxins in this food group, still avoiding time-consuming and laborious sample extraction and extract purification procedures.

This thesis, by developing a fully novel EIA, and by targeting two relevant but largely neglected groups of food products, provides support for the conclusion that EIAs, even 50 years after their first description, still offer interesting fields of application. Most laboratories will not develop EIAs for their specific analytical demands, but will use commercial test kits, which are available for the major EU-regulated toxins. Extra-label use of such tests, similar as demonstrated in **study 3**, may result in false positive results, which in turn could generate problems with the customer, cause unnecessary financial losses, and undermine the trust in

the reliability of routine food control. When used wisely, critically reviewing positive and suspect results, EIAs can still be a valuable, versatile tool in food hygiene.

5 References

Ackermann, Y., Curtui, V., Dietrich, R., Gross, M., Latif H., Märtlbauer, E., & Usleber, E. (2011). Widespread occurrence of low levels of alternariol in apple and tomato products, as determined by comparative immunochemical assessment using monoclonal and polyclonal antibodies. *Journal of Agricultural and Food Chemistry*, *59*, 6360-6368. https://doi.org/10.1021/jf201516f

Acklin, W., F. Weibel, & D. Arigoni (1977). Zur Biosynthese von Paspalin und verwandten Metaboliten aus *Claviceps paspali*. *Chimia, 31*, 63.

Adegbeye, M.J., Reddy, P.R.K., Chilaka, C.A., Balogun, O.B., Elghandour, M.M.M.Y., Rivas-Caceres, R.R., & Salem, A.Z.M. (2020). Mycotoxin toxicity and residue in animal products: prevalence, consumer exposure and reduction strategies–a review. *Toxicon*, *177*, 96-108. https://doi.org/10.1016/j.toxicon.2020.01.007

Andersen, B., & Frisvad, J.C. (2004). Natural occurrence of fungi and fungal metabolites in moldy tomatoes. *Journal of Agricultural and Food Chemistry*, *52*, 7507–7513. https://doi.org/10.1021/jf048727k

Anfossi, L., Baggiani, C., Giovannoli, C., D'Arco, G., & Giraudi, G. (2013): Lateral-flow immunoassays for mycotoxins and phycotoxins: a review. *Analytical and Bioanalytical Chemistry*, *405*, 467-80. https://doi.org/10.1007/s00216-012-6033-4

Aichinger, G., Kruger, F., Puntscher, H., Preindl, K., Warth, B., & Marko, D. (2019). Naturally occurring mixtures of Alternaria toxins: anti-estrogenic and genotoxic effects in vitro. *Archives of Toxicology*, *93*(10), 3021-3031. https://doi.org/10.1007/s00204-019-02545-z

Alshannaq, A., & Yu, J.H. (2017). Occurrence, toxicity, and analysis of major mycotoxins in food. *International Journal of Environmental Research and Public Health*, *14*(6), 632:1-20. https://doi.org/10.3390/ijerph14060632

Al-Taher, F., Cappozzo, J., Zweigenbaum, J., Lee, H.J., Jackson, L., & Ryu, D. (2017). Detection and quantitation of mycotoxins in infant cereals in the U.S. market by LC-MS/MS using a stable isotope dilution assay. *Food Control*, *72*, 27-35. https://doi.org/10.1016/j.foodcont.2016.07.027

Arcella, D., Eskola, M., & Gómez Ruiz, J. A. (2016). Dietary exposure assessment to *Alternaria* toxins in the European population. *EFSA Journal*, *14*(12), 4654. https://doi.org/10.2903/j.efsa.2016.4654

Arroyo-Manzanares, N., Hamed, A.M., García-Campaña, A.M., & Gámiz-Gracia, L. (2019). Plant-based milks: Unexplored source of emerging mycotoxins. A proposal for the control of enniatins and beauvericin using UHPLC-MS/MS. *Food Additives and Contaminants Part B*, *12*, 296-302. https://doi.org/10.1080/19393210.2019.1663276

Assunção, R., Martins, C., Vasco, E., Jager, A., Oliveira, C., Cunha, S.C., Fernandes, J.O., Nunes, B., Loureiro, S., & Alvito, P. (2018). Portuguese children dietary exposure to multiple mycotoxins - An overview of risk assessment under MYCOMIX project. *Food and Chemical Toxicology*, *118*, 399-408. https://doi.org/10.1016/j.fct.2018.05.040

Assunção, R., Boué, G., Alvito, P., Brazão, R., Carmona, P., Carvalho, C., Correia, D., Fernandes, P., Lopes, C., Martins, C., Membré, J.-M., Monteiro, S., Nabais, P., Thomsen, S.T., Torres, D., Pires, S.M., & Jakobsen, L.S. (2021). Risk-benefit assessment of cereal-based foods consumed by Portuguese children aged 6 to 36 months - a case study under the RiskBenefit4EU project. *Nutrients*, *13*(9), 3127. https://doi.org/10.3390/nu13093127

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C., & Forsythe, S. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiology, 9*, 223. http://dx.doi.org/10.1186/1471-2180-9-223

Bar-Oz, B., Preminger, A., Peleg, O., Block, C., & Arad, I. (2001). *Enterobacter sakazakii* infection in the newborn. *Acta Paediatr*, *90*, 356–358. https://doi.org/10.1111/j.1651-2227.2001.tb00319.x

Bauer, J. I., Gross, M., Cramer, B., Humpf, H.-U., Hamscher, G., & Usleber, E. (2018). Immunochemical analysis of paxilline and ergot alkaloid mycotoxins in grass seeds and plants. *Journal of Agricultural and Food Chemistry*, *66*, 315–322. https://doi.org/10.1021/acs.jafc.7b05580

Benkerroum, N. (2020). Chronic and acute toxicities of aflatoxins: mechanisms of action. *International Journal of Environmental Research and Public Health*, *17*(2):423. https://doi.org/10.3390/ijerph17020423

Botha, C. J., Visagie, C. M., & Sulyok, M. (2019). Putative neuromycotoxicoses in an adult male following ingestion of moldy walnuts. *Mycotoxin Research*, *35*, 9–16. https://doi.org/10.1007/s12550-018-0326-1

Braun, D., Eiser, M., Puntscher, H., Marko, D., & Warth, B. (2020). Natural contaminants in infant food: The case of regulated and emerging mycotoxins. *Food Control*, *123*, 107676. https://doi.org/10.1016/j.foodcont.2020.107676

Brugger, E. M., Wagner, J., Schumacher, D. M., Koch, K., Podlech, J., Metzler, M., & Lehmann, L. (2006). Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. Toxicology Letters, 164, 221– 230. https://doi.org/10.1016/j.toxlet.2006.01.001

Bullerman, L., & Bianchini, A. (2007). Stability of mycotoxins during food processing. *International Journal of Food Microbiology*, *119*, 140–146. https://doi.org/10.1016/j.ijfoodmicro.2007.07.035

Canady, R.A., Coker, R.D., Rgan, S.K., Krska, R., Kuiper-Goodman, T., Olsen, M., Pestka, J.J., Resnik, S., & Schlatter, J. (2001). Deoxynivalenol. Safety evaluation of certain mycotoxins in food. Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Food Additives Series 47. International Programme on Chemical Safety–World Health Organization–Geneva, 420–555. Available online: https://www.fao.org/3/bc528e/bc528e.pdf (accessed on 05 August 2022)

Chen, J.-J., Han, M.-Y., Gong, T., Yang, J.-L., & Zhu, P. (2017). Recent progress in ergot alkaloid. *RSC Advances*, *7*(44), 27384–27396. https://doi.org/10.1039/C7RA03152A

Cole, R.J., Kirksey, J.W., Wells, J.M. (1974). A new tremorgenic metabolite of *Penicillium paxilli*. *Canadian Journal of Microbiology*, *20*, 1159–62. https://doi.org/10.1139/m74-179

Cole, R. J., Dorner, J. W., Cox, R. H., & Raymond, L. W. (1983). Two classes of alkaloid mycotoxins produced by *Penicillium crustosum* Thom isolated from contaminated beer. *Journal of Agricultural and Food Chemistry*, 31, 655–657. https://doi.org/10.1021/jf00117a045

Coyle, C.M., & Panaccione, D.G. (2005). An ergot alkaloid biosynthesis gene and clustered hypothetical genes from *Aspergillus fumigatus*. *Applied and Environmental Microbiology*, *71*(6), 3112–3118. https://dx.doi.org/10.1128%2FAEM.71.6.3112-3118.2005

Curtui, V., Usleber, E., Trebstein, A., Lauber, U., Hocke, K., Dietrich, R., Märtlbauer, E., Majerus, P., Zimmer, M., Klaffke, H., Gareis, M., Betsche, T., & Langenkämper, G. (2009). Improvement and validation of analytical methods for type A trichothecenes (T-2 toxin and HT-2 toxin) and occurrence of these mycotoxins in foods from the German market. Bundesanstalt für Landwirtschaft und Ernährung (BLE). Available from: https://service.ble.de/ptdb/index2.php?detail_id=88841&site_key=145&stichw=trichothecene&zeilenzahl_zaehler=4#newContent (accessed on 05 August 2022).

De Jesus, A. E., Gorst-Allman, C. P., Steyn, P. S., van Heerden, F. R., Vleggaar, R., Wessels, P. L., & Hull, W. E. (1983). Tremorgenic mycotoxins from *Penicillium crustosum*. Biosynthesis of Penitrem A. *Journal of the Chemical Society Perkin Transactions 1*, 1863-1868. https://doi.org/10.1039/P19830001863

EC. (2006). Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union, L 364*, 5-24. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF (accessed on 03 August 2022).

EC. (2013). Commission recommendation 2013/165/EU of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. *Official Journal of the European Union, L 91*, 12-15. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013H0165&from=EN (accessed on 03 August 2022).

EC. (2022). Commission recommendation (EU) 2022/553 of 5 April 2022 on monitoring the presence of *Alternaria* toxins in food. *Official Journal of the European Union, L 107/90*. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32022H0553&from=DE (accessed on 03 August 2022).

Edwards, S.G. (2009a). *Fusarium* mycotoxin content of UK organic and conventional oats. Food Additives and Contaminants Part A, 26, 1063-1069. https://doi.org/10.1080/02652030902788953

Edwards, S.G. (2009b). *Fusarium* mycotoxin content of UK organic and conventional barley. Food Additives and Contaminants Part A, 26, 1185-1190. https://doi.org/10.1080/02652030902919418

Edwards, S.G. (2009c). *Fusarium* mycotoxin content of UK organic and conventional wheat. Food Additives and Contaminants Part A, 26, 496-506. https://doi.org/10.1080/02652030802530679

EFSA. (2006). Opinion of the scientific panel on contaminants in the food chain on request from the commission related to ochratoxin A in food. *EFSA Journal, 365*, 1–56. https://doi.org/10.2903/j.efsa.2006.365

EFSA. (2011). Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA Journal, 9*(10), 2407. https://doi.org/10.2903/j.efsa.2011.2407

EFSA. (2013). Scientific opinion on the risk for public and animal health related to the presence of sterigmatocystin in food and feed. *EFSA Journal, 11*, 3254. https://doi.org/10.2903/j.efsa.2013.3254

EFSA. (2017). Human and animal dietary exposure to T-2 and HT-2 toxin. *EFSA Journal, 15*, 4972. https://doi.org/10.2903/j.efsa.2017.4972

Ehling-Schulz, M., Fricker, M., & Scherer, S. (2004). *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. *Molecular Nutrition & Food Research*, *48*, 479–487. https://doi.org/10.1002/mnfr.200400055

Engelhart, S., Loock, A., Skutlarek, D., Sagunski, H., Lommel, A., Färber, H., & Exner, M. (2002). Occurrence of toxigenic *Aspergillus versicolor* isolates and sterigmatocystin in carpet dust from damp indoor environments. *Applied and Environmental Microbiology*, *68*(8), 3886-90. https://doi.org/10.1128%2FAEM.68.8.3886-3890.2002

Eriksen, G.S., Moldes-Anaya, A., & Fæste, C.K. (2013). Penitrem A and analogues: toxicokinetics, toxicodynamics including mechanism of action and clinical significance. *World Mycotoxin Journal*, *6*, 263–272. https://doi.org/10.3920/WMJ2013.1574

Fakruddin, M., Rahaman, M., Ahmed, M.M., & Hoque, M.M. (2014). Stress tolerant virulent strains of *Cronobacter sakazakii* from food, *Biological Research, 47*, 63. http://dx.doi.org/10.1186/0717-6287-47-63.

Fehr, M., Pahlke, G., Fritz, J., Christensen, M.O., Boege, F., Altemöller, M., Podlech, J., & Marko, D. (2009). Alternariol acts as a topoisomerase poison, preferentially affecting the IIalpha isoform. *Molecular Nutrition and Food Research, 53*, 441–451. https://doi.org/10.1002/mnfr.200700379

Finch, S.C., Thom, E.R., Babu, J.V., Hawkes, A.D., & Waugh, C.D. (2013). The evaluation of fungal endophyte toxin residues in milk. *New Zealand Veterinary Journal*, *61*, 11–17. https://doi.org/10.1080/00480169.2012.704626

Gordon, K. E., Masotti, R. E., & Waddell, W. R. (1993). Tremorgenic encephalopathy: A role of mycotoxins in the production of CNS disease in humans? *Canadian Journal of Neurological Sciences*, *20*, 237–239. https://doi.org/10.1017/S0317167100048010

Gotthardt, M., Asam, S., Gunkel, K., Moghaddam, A.F., Baumann, E., Kietz, R., & Rychlik, M. (2019). Quantitation of six *Alternaria* toxins in infant foods applying stable isotope labeled standards. *Frontiers in Microbiology*, *10*, 109. https://doi.org/10.3389/fmicb.2019.00109

Gottschalk, C., Barthel, J., Engelhardt, G., Bauer, J. & Meyer, K. (2007). Occurrence of type A trichothecenes in conventionally and organically produced oats and oat products. *Molecular Nutrition & Food Research, 51*, 1547-1553. https://doi.org/10.1002/mnfr.200700146

Gottschalk, C., Barthel, J., Engelhardt, G., Bauer, J., & Meyer, K. (2009). Simultaneous determination of type A, B and D trichothecenes and their occurrence in cereals and cereal products. Food Additives and Contaminants *Part A*, 26, 1273-1289. https://doi.org/10.1080/02652030903013260

Gross, M., Curtui, V., & Usleber, E. (2018). Detection of total ergot alkaloids in cereal flour and in bread by a generic enzyme immunoassay method. *Journal of AOAC International*, *101*, 618–626. https://doi.org/10.5740/jaoacint.17-0346

Hamed, A.M., Arroyo-Manzanares, N., García-Campaña, A.M., & Gámiz-Gracia, L. (2017) Determination of Fusarium toxins in functional vegetable milks applying salting out assisted liquid-liquid extraction combined with ultra-high performance liquid chromatography tandem mass spectrometry. *Food Additives and Contaminants Part A*, *34*, 2033-2041. https://doi.org/10.1080/19440049.2017.1368722

Hamed, A.M., Abdel-Hamid, M., Gámiz-Gracia, L., García-Campaña, A.M., Arroyo-Manzanares, N. (2019). Determination of aflatoxins in plant-based milk and dairy products by dispersive liquid-liquid microextraction and high- performance liquid chromatography with fluorescence detection. *Analytical Letters*, *52*, 363-372. http://dx.doi.org/10.1080/00032719.2018.1467434

He, T., Zhu, J., Nie, Y., Hu, R., Wang, T., Li, P., Zhang, Q., & Yang, Y. (2018): Nanobody technology for mycotoxin detection in the field of food safety: current status and prospects. *Toxins*, *10*(5), 180. https://doi.org/10.3390/toxins10050180

Herrera, M., Bervis, N., Carramiñana, J. J., Juan, T., Herrera, A., Ariño, A., & Lorán, S. (2019). Occurrence and exposure assessment of aflatoxins and deoxynivalenol in cereal-based baby foods for infants. *Toxins, 11*, 150. https://doi.org/10.3390/toxins11030150

IARC. (1987). IARC Monographs on the evaluation of carcinogenic risks to humans. Summaries and evaluations, sterigmatocystin. Suppl. 7, 72. Available online: https://monographs.iarc.who.int/wp-content/uploads/2018/06/Suppl7.pdf (accessed on 05 August 2022)

IARC. (1993). Monographs on the evaluation of carcinogenic risks to humans: some naturally occuring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, 56, 489–524. Available online: https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Naturally-Occurring-Substances-Food-Items-And-Constituents-Heterocyclic-Aromatic-Amines-And-Mycotoxins-1993 (accessed on 05 August 2022)

IARC. (2012). Monographs on the evaluation of carcinogenic risks to humans: chemical agents and related occupations. A review of human carcinogens, 100F, 224–248. Available online: https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Chemical-Agents-And-Related-Occupations-2012 (accessed on 05 August 2022)

ICMSF. (2011). Microorganisms in Foods 8: Use of Data for Assessing Process Control and Product Acceptance (1st ed). Springer. https://doi.org/10.1007/978-1-4419-9374-8_15

Imlach, W.L., Finch, S.C., Zhang, Y., Dunlop, J., & Dalziel, J.E. (2011). Mechanism of action of lolitrem B, a fungal endophyte derived toxin that inhibits BK large conductance Ca^{2+}-activated K^+ channels. *Toxicon, 57*, 686–694. https://doi.org/10.1016/j.toxicon.2011.01.013

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R., & Joosten, H. (2008). Cronobacter gen. nov., a new genus to accommodate the biogroups of Enterobacter sakazakii, and proposal of Cronobacter sakazakii gen. nov., comb. nov.,Cronobacter malonaticus sp. nov.,Cronobacter turicensis sp. nov.,Cronobacter muytjensii sp. nov.,Cronobacter dublinensis sp. nov.,Cronobacter genomospecies 1, and of three subspecies, Cronobacter dublinensis subsp. Dublinensis subsp. nov.,Cronobacter dublinensis subsp. lausannensis subsp. nov. and Cronobacter dublinensis subsp. lactaridi subsp. nov. *International Journal of Systematic and Evolutionary Microbiology, 58*, 1442–1447. https://doi.org/10.1099/ijs.0.65577-0

Janik, E., Niemcewicz, M., Podogrocki, M., Ceremuga, M., Gorniak, L., Stela, M., & Bijak, M. (2021). The existing methods and novel approaches in mycotoxins' detection. *Molecules*, *26*(13), 3981. https://doi.org/10.3390/molecules26133981

Jaradat, Z.W. (2005). T-2 mycotoxin in the diet and its effects on tissues. In: Watson, R.R. and Preedy, V.R. (eds.) Reviews in food and nutrition toxicity. Vol. 4. CRC Press, Boca Raton, FL, USA, 173-212.

Juan, C., Raiol, A., Mañes, J., & Ritieni, A. (2014). Presence of mycotoxin in commercial infant formulas and baby foods from Italian market. *Food Control*, *39*, 227-236. http://dx.doi.org/10.1016/j.foodcont.2013.10.036

Juan, C., Mañes, J., Juan-García, A., & Moltó J.C. (2022). Multimycotoxin analysis in oat, rice, almond and soy beverages by liquid chromatography-tandem mass spectrometry. *Applied Sciences*, *12*, 3942. https://doi.org/10.3390/app12083942

Kalinina, S. A., Jagels, A., Hickert, S., Mauriz Marques, L. M., Cramer, B., & Humpf, H.-U. (2018). Detection of the cytotoxic penitrems A−F in cheese from the European single market by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 66*, 1264-1269. https://doi.org/10.1021/acs.jafc.7b06001

Kanik T, & Kabak B (2019). Aflatoxins in almonds: Monitoring and exposure assessment. *Journal of Food Safety*, *39*, e12646. http://dx.doi.org/10.1111/jfs.12646

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I.P., Speijers, G., Chiodini, A., Recker, T., & Dussort, P. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin Research, 32*, 179-205. https://doi.org/10.1007/s12550-016-0257-7

Kim, S.A., Oh, S.W., Lee, Y.M., Imm, J.Y., Hwang, I.G., Kang, D.H., & Rhee, M.S. (2011). Microbial contamination of food products consumed by infants and babies in Korea. *Letters in Applied Microbiology*, *53*, 532-538. https://doi.org/10.1111/j.1472-765X.2011.03142.x

King, A.D., & Schade, J.E. (1984). *Alternaria* toxins and their importance in food. *Journal of Food Protection*, *47*, 886–901. https://doi.org/10.4315/0362-028x-47.11.886

Knaus, H. G., McManus, O. B., Lee, S. H., Schmalhofer, W. A., Garcia-Calvo, M., Helms, L. M., Sanchez, M., Giangiacomo, K., & Reuben, J. P. (1994). Tremorgenic indole alkaloids potently

inhibit smooth muscle high-conductance calcium-activated potassium channels. Biochemistry, 33, 5819–5828. https://doi.org/10.1021/bi00185a021

Kramer, J.M., & Gilbert, R.J. (1989). *Bacillus cereus* and other *Bacillus* species. In: Foodborne bacterial pathogens (Doyle, M.P., Ed.), 21-70. Marcel Dekker, New York.

Krska, R., Malachova, A., Berthiller, F., & van Egmond, H.P. (2014). Determination of T-2 and HT-2 toxins in food and feed: An update. *World Mycotoxin Journal*, *7*, 131–142. https://doi.org/10.3920/WMJ2013.1605

Kucerova, E., Clifton, S.W., Xia, X.Q., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P.,Kyung, K., Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash,W.E., Hallsworth-Pepin, K., Wilson, R.K., McClelland, M., & Forsythe, S.J. (2010).Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. *PLoS One*, *5*, e9556. https://doi.org/10.1371/journal.pone.0009556

Kuiper-Goodman, T., Scott, P. M., & Watanabe, H. (1987). Risk assessment of the mycotoxin zearalenone. *Regulatory Toxicology and Pharmacology*, *7*, 253–306. https://doi.org/10.1016/0273-2300(87)90037-7

Kumar, A., Pathak, H., Bhadauria, S., & Sudan, J. (2021). Aflatoxin contamination in food crops: causes, detection, and management: a review. *Food Production Processing and Nutrition, 3*, 17. https://doi.org/10.1186/s43014-021-00064-y

Lewis, P. R., Donoghue, M. B., Hocking, A. D., Cook, L., & Granger, L. V. (2005). Tremor syndrome associated with a fungal toxin: Sequelae of food contamination. *Medical Journal of Australia*, *182*, 582–584. https://doi.org/10.5694/j.1326-5377.2005.tb06819.x

Liesener, K., Curtui, V., Dietrich, R., Märtlbauer, E., & Usleber, E. (2010). Mycotoxins in horse feed. *Mycotoxin Research, 26*, 23-30. https://doi.org/10.1007/s12550-009-0037-8

Logrieco, A., Moretti, A., & Solfrizzo, M. (2009). *Alternaria* toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin Journal, 2*, 129–140. http://dx.doi.org/10.3920/WMJ2009.1145

Los, A., Ziuzine, D., & Bourke, P. (2018). Current and future technologies for microbiological decontamination of cereal grains. *Journal of Food Science*, *83*(6), 1484-149. https://doi.org/10.1111/1750-3841.14181

Luo, X.Y. (1994). Food poisoning caused by *Fusarium* toxins. In: Proceedings of the Second Asian Conference on Food Safety, Chatuchak, Bangkok, Thailand; International Life Sciences Institute: Washington, DC, USA, 129–136.

Lutsky, I., Mor, N., Yagen, B., & Joffe, A.Z. (1978). The role of T-2 toxin in experimental alimentary aleukia, a toxicity study in cats. *Toxicology and Applied Pharmacology*, *43*, 111-124. https://doi.org/10.1016/S0041-008X(78)80036-2

Malir, F., Ostry, V., & Novotna, E. (2013). Toxicity of the mycotoxin ochratoxin A (OTA) in the light of recent data. *Toxin Reviews*, *32*(2). http://dx.doi.org/10.3109/15569543.2013.782504

Malysheva, S.V., Larionova, D.A., Di Mavungu, J.D.D., & De Saeger, S. (2014). Pattern and distribution of ergot alkaloids in cereals and cereal products from European countries. *World Mycotoxin Journal, 7*, 217–230. https://doi.org/10.3920/WMJ2013.1642

Mannich, C., & Krösche, W. (1912). Über ein Kondensationsprodukt aus Formaldehyd, Ammoniak und Antipyrin. *Archiv der Pharmazie, 250*(1), 647–667. https://doi.org/10.1002/ardp.19122500151

Mantle, P. G., & Penn, J. (1989). A role for paxilline in the biosynthesis of indole- diterpenoid penitrem mycotoxins. Journal of the Chemical Society Perkin Transactions 1, 1539–1540. https://doi.org/10.1039/P19890001539

Marin, S., Ramos, A.J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology, 60*, 218–237. https://doi.org/10.1016/j.fct.2013.07.047

Mashoufi, A., Hashemi, M., Ghazvini, K., Mobarhan, M.G. & Afshari, A. (2017). *Cronobacter sakazakii*, a new threat: Characteristic, molecular epidemiology and virulence factors. *Annual Research and Review in Biology*, 1-21. https://doi.org/10.9734/ARRB/2017/37657

Messelhäußer, U., & Ehling-Schulz, M. (2018). *Bacillus cereus*-a multifaceted opportunistic pathogen. *Current Clinical Microbiology Reports, 5*, 120–125. https://doi.org/10.1007/s40588-018-0095-9

Miller, J. D., & Trenholm, H. L. (editors), 1994, Mycotoxins in Grain: Compounds Other Than Aflatoxin, St Paul, Eagan Press.

Miró-Abella, E., Herrero, P., Canela, N., Arola, L., Borrull, F., Ras, R., & Fontanals, N. (2017). Determination of mycotoxins in plant-based beverages using QuEChERs and liquid chromatography-tandem mass spectrometry. *Food Chemistry, 229*, 336-372. https://doi.org/10.1016/j.foodchem.2017.02.078

Mirocha, C. J., & Christensen, C.M. (1974). Oestrogenic mycotoxins synthesized by *Fusarium*. In: Mycotoxins, pp. 129 148. In I. F. H. Purchase (ed.), Mycotoxins. Elsevier, Amsterdam, The Netherlands.

Mol, H.G., Mac Donald, S., Anagnostopoulos, C., Spanjer, M., Bertuzzi, T., & Pietri, A. (2016). European survey on sterigmatocystin in cereals, cereals-based products, beer and nuts. *World Mycotoxin Journal, 9*, 633–642. http://dx.doi.org/10.3920/WMJ2016.2062

Müller, A., Stephan, R., Fricker-Feer, C., & Lehner, A. (2013).Genetic diversity of *Cronobacter sakazakii* isolates collected from a Swiss infant formula production facility. *Journal of Food Protection, 76*, 883–887. https://doi.org/10.4315/0362-028x.jfp-12-521

Ostry, V. (2008). Alternaria mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal*, *1*, 175-188. http://dx.doi.org/10.3920/WMJ2008.x013

Oueslati, S., Berrada, H., Mañes, J., & Juan, C. (2018). Presence of mycotoxins in Tunisian infant foods samples and subsequent risk assessment. *Food Control, 84*, 362-369. https://doi.org/10.1016/j.foodcont.2017.08.021

Ozcelik, S., Ozcelik, N., & Beuchat, L.R. (1990). Toxin production by *Alternaria alternata* in tomatoes and apples stored under various conditions and quantitation of the toxins by high-performance liquid chromatography. *International Journal of Food Microbiology, 11*, 187–194. https://doi.org/10.1016/0168-1605(90)90011-s

Panaccione, D.G. (2005). Origins and significance of ergot alkaloid diversity in fungi. *FEMS Microbiology Letters, 251*, 9–17. https://doi.org/10.1016/j.femsle.2005.07.039

Pecorelli, I., Guarducci, N., von Holst, C., Bibi, R., Pascale, M., Ciasca, B., Logrieco, A.F., & Lattanzio, V.M.T. (2020). Critical comparison of analytical performances of two immunoassay methods for rapid detection of aflatoxin M_1 in milk. *Toxins, 12*, 270. https://doi.org/10.3390/toxins12040270

Penagos-Tabares, F., Khiaosa-ard, R., Nagl, V., Faas, J., Jenkins, T., Sulyok, M., & Zebeli, Q. (2021). Mycotoxins, phytoestrogens and other secondary metabolites in Austrian pastures: Occurrences, contamination levels and implications of geo-climatic factors. *Toxins, 13*, 460. https://doi.org/10.3390/toxins13070460

Pereira, V.L., Fernandes, J.O., & Cunha, S.C. (2015). Comparative assessment of three cleanup procedures after QuEChERS extraction for determination of trichothecenes (type A and type B) in processed cereal-based baby foods by GC-MS. *Food Chemistry, 182*, 143-149. https://doi.org/10.1016/j.foodchem.2015.01.047

Pestka, J.J. (2008). Mechanisms of deoxynivalenol-induced gene expression and apoptosis. *Food Additives and Contaminants Part A, 25*(9), 1128-40. https://doi.org/10.1080%2F02652030802056626

Pierri, L., Pitman, I.H., Rae, I.D., Winkler, D.A., & Andrews, P.R. (1982). Conformational analysis of the ergot alkaloids ergotamine and ergotaminine. Journal of Medicinal Chemistry, 137, 937–942. https://doi.org/10.1021/jm00350a010

Pitt, J., & Hocking, A. (2009). Fungi and Food Spoilage. Blackie Academic and Professional, London. http://dx.doi.org/10.1007/978-0-387-92207-2

Poor, M., Kunsagi-Mate, S., Sali, N., Koszegi, T., Szente, L., & Peles-Lemli, B. (2015). Interactions of zearalenone with native and chemically modified cyclodextrins and their potential utilization. *Journal of Photochemistry and Photobiology B, 151*, 63–68. https://doi.org/10.1016/j.jphotobiol.2015.07.009

Reddy, P., Guthridge, K., Vassiliadis, S., Hemsworth, J., Hettiarachichige, I., Spangenberg, G., & Rochfort, S. (2019). Tremorgenic mycotoxins: structure diversity and biological activity. *Toxins*, *11*, 302. https://doi.org/10.3390/toxins11050302

Richard, J. L., Bacchetti, P., & Arp, L. H. (1981). Moldy walnut toxicosis in a dog, caused by the mycotoxin, penitrem A. *Mycopathologia, 76*, 55–58. https://doi.org/10.1007/bf00761899

Rosenstein, Y., Lafarge-Frayssinet, C., Lespinats, G., Loisillier, F., Lafont, P., & Frayssinet, C. (1979). Immunosuppressive activity of *Fusarium* toxins. Effects on antibody synthesis and skin grafts of crude extracts, T2-toxin and diacetoxyscirpenol. *Immunology, 36*, 111-117. http://www.ncbi.nlm.nih.gov/pmc/articles/pmc1457389/

Rundberget, T., & Wilkins, A. L. (2002). Determination of *Penicillium* mycotoxins in foods and feeds using liquid chromatography−mass spectrometry. *Journal of Chromatography A, 964*, 189−197. https://doi.org/10.1016/s0021-9673(02)00698-2

Rundberget, T., Skaar, I., & Flåøyen, A. (2004). The presence of *Penicillium* and *Penicillium* mycotoxins in food wastes. *International Journal of Food Microbiology, 90*, 181–188. https://doi.org/10.1016/s0168-1605(03)00291-5

Rychlik, M., Humpf, H.-U., Marko, D., Dänicke, S., Mally, A., Berthiller, F., Klaffke, H., & Lorenz, N. (2014). Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. *Mycotoxin Research, 30*, 197-205. https://doi.org/10.1007%2Fs12550-014-0203-5

Saikia, S., Nicholson, M.J., Young, C., Parker, E.J., & Scott, B. (2008). The genetic basis for indole diterpene chemical diversity in filamentous fungi. *Mycological Research, 112*, 184–199. https://doi.org/10.1016/j.mycres.2007.06.015

Santos Pereira, C., C. Cunha, S., & Fernandes, J.O. (2019). Prevalent mycotoxins in animal feed: occurrence and analytical methods. *Toxins*, *11*, 290. https://doi.org/10.3390/toxins11050290

Scott, P. M, Van Walbeek, W., Kennedy, B., & Anyeti, D. (1972). Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. *Journal of Agricultural and Food Chemistry, 20*, 1103–1109. https://doi.org/10.1021/jf60184a010

Scott, P.M. (1981). Toxins of *Penicillium* species used in cheese manufacture. *Journal of Food Protection, 44*, 702-710. https://doi.org/10.4315/0362-028x-44.9.702

Scott, P.M. (2001). Analysis of agricultural commodities and foods for *Alternaria* mycotoxins. *Journal of AOAC International, 84*, 1809-1817. https://doi.org/10.1093/jaoac/84.6.1809

Scott, P.M., Zhao, W., Feng, S., & Lau, B.P. (2012). *Alternaria* toxins alternariol and alternariol monomethyl ether in grain foods in Canada. *Mycotoxin Research, 28*, 261-6. https://doi.org/10.1007/s12550-012-0141-z

Smith, B.L., McLeay, L.M., & Embling, P.P. (1997). Effect of the mycotoxins penitrem, paxilline and lolitrem B on the electromyographic activity of skeletal and gastrointestinal smooth muscle of sheep. *Research in Veterinary Science, 62*, 111–116. https://doi.org/10.1016/s0034-5288(97)90130-2

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., & Kizek, R. (2010). Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology, 3*, 94–99. https://doi.org/10.2478%2Fv10102-010-0019-x

Stenfors Arnesen, L.P., Fagerlund, A., & Granum, P.E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews*, *32*, 579–606. https://doi.org/10.1111/j.1574-6976.2008.00112.x

Stoev, S.D. (2022). New evidences about the carcinogenic effects of ochratoxin A and possible prevention by target feed additives. *Toxins, 14,* 380. https://doi.org/10.3390/toxins14060380

Tanaka, T., Hasegawa, A., Yamamoto, S., Soo Lee, U., Sugiura, Y., & Ueno, Y. (2002). Worldwide contamination of cereals by the *Fusarium* mycotoxins nivalenol, deoxynivalenol, and zearalenone. 1. Survey of 19 countries. Journal of Agricultural and Food Chemistry, 36, 979–983. https://doi.org/10.1021/jf00083a019

Tor, E.R., Puschner, B., Filigenzi, M.S., Tiwary, A.K., & Poppenga, R.H. (2006): LC-MS/MS screen for PTA and roquefortine C in serum and urine samples. Analytical Chemistry, 78, 4624-4629. https://doi.org/10.1021/ac0601617

Townsend, S., & Forsythe, S.J. (2008). The neonatal intestinal microbial flora, immunity, and infections. In: *Enterobacter sakazakii* ed. Farber, J. and Forsythe, S.J. pp. 61–100 Washington, DC: ASM Press.

Ueno, Y. (1984). Toxicological features of T-2 toxin and related trichothecenes. *Fundamental and Applied Toxicology, 4*, S124–S132.

Van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A., & Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology, 39*, 293–297. https://doi.org/10.1128/jcm.39.1.293-297.2001

Vandicke, J., De Visschere, K., Ameye, M., Croubels, S., De Saeger, S., Audenaert, K., & Haesaert, G. (2021). Multi-mycotoxin contamination of maize silages in Flanders, Belgium: monitoring mycotoxin levels from seed to feed. *Toxins, 13*, 202. https://doi.org/10.3390/toxins13030202

Vesonder, R.F., Ciegler, A., & Jensen, A.H. (1973). Isolation of the emetic principle from *Fusarium*-infected corn. *Applied Microbiology, 26*, 1008–1010. https://doi.org/10.1128%2Fam.26.6.1008-1010.1973

Vilain, S., Luo, Y., Hildreth, M.B., & Brözel, V.S. (2006). Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. *Applied and Environmental Microbiology, 72*, 4970–4977. https://doi.org/10.1128%2FAEM.03076-05

Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S. B., Klaassen, C. H. W., Perrone, G., Seifert, K. A., Varga, J., Yaguchi, T., & Samson, R. A. (2014). Identification and nomenclature of the genus *Penicillium*. *Studies in Mycology, 78*, 343–371. https://doi.org/10.1016/j.simyco.2014.09.001

Wong, J.J., Singh, R., & Hsieh, D.P.H. (1977). Mutagenicity of fungal metabolites related to aflatoxin biosynthesis. *Mutation Research, 44*, 447–450. https://doi.org/10.1016/0027-5107(77)90102-6

Young, K. L., Villar, D., Carson, T. L., Imerman, P. M., Moore, R. A., & Bottoff, M. R. (2003). Tremorgenic mycotoxin intoxication with penitrem A and roquefortine in two dogs. *Journal of the American Veterinary Medical Association, 222,* 52-53. https://doi.org/10.2460/javma.2003.222.52

Zhang, K., Flannery, B.M., Oles, C.J., & Adeuya, A. (2018). Mycotoxins in infant/toddler foods and breakfast cereals in the U.S. retail market. *Food Additives and Contaminants Part B*, *11*, 183-190. https://doi.org/10.1080/19393210.2018.1451397

Zheng, M.Z., Richard, J.L., & Binder, J. (2006). A review of rapid methods for the analysis of mycotoxins. *Mycopathologia, 161*, 261–273. https://doi.org/10.1007/s11046-006-0215-6

Ziver, T., Okburan, G., Akgül, Ö., Sarıbaş, S., & Kocazeybek, B. (2020). Investigation of *Cronobacter sakazakii (Enterobacter sakazakii)* presence in cereal infant foods. *Progress in Nutrition, 22*, 596-602. https://doi.org/10.23751/pn.v22i2.8907

6 Publications

6.1 Development and application of a competitive enzyme immunoassay for the detection of penitrem A in fungal mycelium: Evidence for frequent occurrence of multiple indole-containing mycotoxins in mouldy foods

Rehagel C, Akineden Ö, Geisen R, Cramer B, Plötz M, Usleber E

Food Control 132 (2022) 108558

Author contributions:

Rehagel C: Conceptualization, validation, formal analysis, investigation, writing – original draft, writing - review & editing, visualization. **Akineden Ö**: Investigation, supervision, writing -review & editing. **Geisen R**: Investigation, writing - review & editing. **Cramer B**: Investigation, writing - review & editing. Plötz M: Funding acquisition, writing - review & editing. **Usleber E**: Funding acquisition, resources, supervision, project.

Food Control 132 (2022) 108558

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Development and application of a competitive enzyme immunoassay for the detection of penitrem A in fungal mycelium: Evidence for frequent occurrence of multiple indole-containing mycotoxins in mouldy foods

Christina Rehagel [a,*], Ömer Akineden [a], Rolf Geisen [b], Benedikt Cramer [c], Madeleine Plötz [d], Ewald Usleber [a]

[a] Dairy Sciences, Institute of Veterinary Food Science, Justus-Liebig-University Giessen, Ludwigstrasse 21, 35390, Giessen, Germany
[b] Retired from Department of Safety and Quality of Fruits and Vegetables, Max-Rubner-Institute, Haid-und-Neu-Strasse 9, 76121, Karlsruhe, Germany
[c] Institute of Food Chemistry, Westfälische Wilhelms-Universität Münster, Corrensstrasse 45, Münster, 48149, Germany
[d] Institute for Food Quality and Food Safety, University of Veterinary Medicine Hannover, Foundation, Bischofsholer Damm 15, 30173, Hannover, Germany

ARTICLE INFO

Keywords:
Penicillium
Tremorgenic
Indole-diterpene alkaloids
Penitrem A
Food
Immunoassay

ABSTRACT

The present study aimed to develop a novel enzyme immunoassay (EIA) for the tremorgenic mycotoxin, penitrem A (PTA). This competitive EIA, based on rabbit anti-PTA polyclonal antibodies and a PTA-bovine serum albumin conjugate, yielded a mean standard curve detection limit of 220 ng/mL. The PTA-EIA, together with two EIAs for paxilline (PAX) and ergoline alkaloids, were used to study the toxin profiles in culture of fungi isolated from some naturally infected food sample materials, including dairy products, citrus fruits, and walnuts. PTA was produced by eight out of 27 mycelium extracts from malt extract agar and Sabouraud glucose chloramphenicol selective agar (2–200 μg/mL). Ten isolates from all types of food were also positive in the PAX-EIA (0.004–4 μg/mL). The EIA for ergoline alkaloids yielded positive results (0.0002–0.4 μg/mL) in isolates from dairy products and from walnuts, but not from citrus fruits. Control analyses of selected fungal extracts by HPLC-MS/MS for PTA and PAX qualitatively confirmed the EIA results, poor quantitative agreement could be attributed to the presence of penitrems other than PTA, and to PAX analogues, respectively. Selected PTA-positive fungal isolates were subjected to sequencing of the internal transcribed spacer region and the β-tubulin gene, and were identified as *Penicillium polonicum* and *P. crustosum*. In conclusion, *Penicillium* spp. capable to produce PTA, PAX, and ergoline alkaloids, either alone or in combination, appear to be quite common as contaminants on foods at the retail or production level. Some are capable to produce PTA in culture at high levels, which would have to be considered as toxic if present in food.

1. Introduction

Penitrem A (PTA, synonym: tremortin A) is the most important member of the penitrems (De Jesus et al., 1981, 1983a, 1983b, 1983c), a group of at least seven (A-G) tremorgenic mycotoxins within the large class of indole-diterpene alkaloids (González et al., 2003; Reddy et al., 2019). PTA production has most frequently been reported for isolates of *Penicillium crustosum* (Wilson et al., 1968, 1972), but was also found for isolates of *P. palitans* (Hou et al., 1971), *P. carneum* (Boysen et al., 1996), and *P. simplicissimum* (Hayashi et al., 1993). In a study, which aimed at identification of natural compounds with anti-cancer activity, production of penitrems was reported for a marine-derived isolate of *Penicillium*, *P. commune* GS20 (Sallam et al., 2013). With respect to food control, *P. crustosum* is of particular importance as a PTA producer, because it is a widespread and frequent spoilage microorganism in food and feed (Eriksen et al., 2010; Hayes et al., 1976; Hocking et al., 1988; Naudé et al., 2002; Richard et al., 1981; Rundberget, Skaar, & Flåøyen, 2004; Young et al., 2003). *P. polonicum* often occurs in the same habitat as *P. crustosum* but is not known as a typical PTA producer (Santini et al., 2014). Little is known about the occurrence of PTA-producing fungi and the frequency of PTA contamination in foods at the retail level.

Suspected cases of PTA poisoning in humans and in pet or livestock animals have been reported sporadically, most frequently related to mouldy walnuts. Reported cases of presumptive PTA poisoning in

* Corresponding author.
E-mail address: Christina.Rehagel@vetmed.uni-giessen.de (C. Rehagel).

https://doi.org/10.1016/j.foodcont.2021.108558
Received 21 May 2021; Received in revised form 10 September 2021; Accepted 13 September 2021
Available online 15 September 2021

humans after ingestion of contaminated foods had been characterised by vomiting, nausea, severe tremor and bloody diarrhoea, but a clear causal relationship between PTA intake and clinical symptoms could not be established in any of these cases (Botha et al., 2019; Cole et al., 1983; Gordon et al., 1993; Lewis et al., 2005). PTA intoxication most frequently occurs in dogs, because of a distinct preference for rotten food and their easy access to food waste. Recently, Uhlig et al. (2020) detected eleven phase I metabolites of PTA in blood plasma from a dog which was exposed to PTA. The main symptoms observed in this dog were seizures, mydriasis, nystagmus, opisthotonus, signs of depression, posterior paresis and reduced reflexes. These authors also estimated that dietary PTA has a high bioavailability in dogs of more than 50%. Dogs also appear to be very sensitive towards PTA, as little as 0.175 mg/kg body weight was sufficient to induce muscle tremors (Hayes et al., 1976; Hocking et al., 1988; Munday et al., 2008). Neurotoxic symptoms (staggers) linked to PTA in pasture grass have also been observed in farm animals (Mantle & Penny, 1981). Investigations on the toxic potential in mice revealed oral tremorgenic doses between approximately 0.25 mg/kg body weight (Hou et al., 1971) and 0.5 mg/kg body weight (Moldes-Anaya et al., 2012), the oral LD_{50} in mice was at 1.1 mg/kg body weight (Ueno & Ueno, 1978). After uptake of the mycotoxin into the organism, it causes pathological changes in various organs (Hayes et al., 1976) and affects both the central and the peripheral nervous system of mammals in various ways. The exact mechanism of action of the mycotoxin has not been fully elucidated, but several effects have been identified in various studies that are believed to contribute to the neurotoxic effect of PTA and indole-diterpene alkaloids in general. In addition to its ability to block calcium-activated potassium channels (Knaus et al., 1994), PTA also increases the spontaneous release of some neurotransmitters (glutamate, gamma-amino-butyric acid, aspartate) from cerebrocortic synaptosomes (Cavanagh et al., 1998; Norris et al., 1980). Finally, PTA, due to its lipophilic character, is able to cross the blood-brain barrier and enter the central nervous system (Eriksen et al., 2010). On the other hand, modified penitrems such as 6-bromopenitrems B and E showed good antiproliferative, antimigratory and anti-invasive properties against human breast cancer cells *in vitro* and could therefore be of interest for the development of new drugs (Kozák et al., 2019; Sallam et al., 2013).

Currently, there are no regulations for maximum levels of penitrems in foods and feeds, and therefore neither PTA nor other penitrems are usually included in routine mycotoxin control of foods. With the exception of PTA, analytical toxin standards are not available for penitrems. Finally, rapid testing systems such as enzyme immunoassay (EIA) are not commercially available for any tremorgenic indole-diterpene mycotoxins. These factors all may contribute to the limited number of published data on penitrem frequency and levels in foods and feeds. Various physicochemical methods, mostly liquid chromatography with mass spectrometric detection have been described for the determination of PTA in food and other sample materials (Kalinina et al., 2018; Naudé et al., 2002; Rundberget, Skaar, O'Brien, & Flåøyen, 2004), but also thin-layer chromatography and gas chromatography (Braselton & Johnson, 2003; Braselton & Rumler, 1996; Hocking et al., 1988). None of these methods so far is applied to food analysis on a larger, routine scale, which may be due both to high analytical costs and to a lack of awareness concerning the potential presence of tremorgenic toxins. In such a situation, rapid immunoassays may be useful tools to enhance analytical strategies specifically for routine screening and food surveys. In contrast to many other mycotoxins, antibody production or immunochemical detection methods have not yet been described for PTA or any other penitrem. This study therefore aimed at developing a straightforward approach to produce anti-PTA antibodies for use in a competitive EIA method. This EIA was then implemented in an immunochemical strategy to study the potential of spoilage fungi to produce PTA and some other indole alkaloids. Finally, the plausibility of the EIA results was evaluated by comparison with LC-MS/MS, and by characterisation of some of the PTA-producing fungi.

2. Materials and methods

2.1. Chemicals, reagents and buffers

PTA (molecular weight: 634.21 g/mol) was purchased from Fermentek Ltd. (Jerusalem, Israel). Paxilline (PAX) was obtained from Enzo Life Sciences (Lörrach, Germany). Lolitrem B was from AgResearch (Hamilton, New Zealand). Terpendole E was supplied from Abcam (Cambridge, UK). Verruculogen was purchased from Cayman Chemicals (Hamburg, Germany). Ergometrine (ergonovine), glucose oxidase (GOx, molecular weight: 160,000 g/mol) from *Aspergillus niger*, 3,3′,5,5′-tetramethyl benzidine (TMB), dimethyl sulfoxide (DMSO), casein sodium salt and Tween 20 were from Sigma-Aldrich (Taufkirchen, Germany). Keyhole limpet hemocyanin (KLH, molecular weight: 3,000,000 g/mol), formaldehyde solution 37%, casein-peptone soymeal-peptone (CASO) broth, methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Swine anti-rabbit IgG-horseradish peroxidase (HRP) was supplied from Dako (Hamburg, Germany). Malt extract agar (MEA) and Sabouraud glucose chloramphenicol (0.1 g/L) selective agar (SA) were purchased from Oxoid (Wesel, Germany). Malt extract glucose agar (MG) and yeast extract sucrose agar (YES) were obtained from Carl Roth (Karlsruhe, Germany). All reagents used were at least of analytical grade. A reference strain, *P. crustosum* Thom DSM 62837, formerly isolated from a rotting fruit of *Citrus maxima*, was obtained from DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The concentration and purity of the PTA standard solution (1.0 mg/mL in methanol) was checked by UV-spectroscopy (UV-VIS; Shimadzu, Duisburg, Germany). Spectra for PTA standard solutions in methanol (2.5 μg/mL, 5 μg/mL, 10 μg/mL), which were in full agreement with published data, with absorbance maxima at 233 nm (ε = 37,000 L × mol^{-1} × cm^{-1}) and 295 nm (ε = 11,600 L × mol^{-1} × cm^{-1}) (De Jesus et al., 1983a), were used for quantification. For EIA, MaxiSorp microtiter plates from Nunc (Roskilde, Denmark) were coated with solid phase antigen diluted in sodium bicarbonate buffer (1.59 g Na_2CO_3, $NaHCO_3$ 2.93 g in 1 L distilled water; 0.05 mol/L, pH 9.6). Blocking solution contained 2% of casein sodium salt in phosphate-buffered saline (PBS; 6.79 g NaCl, 1.47 g Na_2HPO_4, 0.43 g KH_2PO_4 in 1 L of distilled water; 0.01 mol/L, pH 7.2). Washing solution contained 8.5 g NaCl and 0.25 mL of Tween in 1 L distilled water. PTA standard solution was prepared in 10% methanol/PBS. For dilution of swine anti-rabbit IgG-HRP PBS was used. For the enzyme substrate/chromogen solution, H_2O_2-citrate buffer (8.3 g citric acid, 49 mL KOH (1 mol/L), 72 μL 30% aqueous H_2O_2, 160 mL distilled water; pH 3.9) and TMB solution (50.4 mg 3,3′,5,5′-TMB, 1 mL acetone, 9 mL methanol) were used. The enzyme substrate/chromogen solution was prepared according to Ackermann et al. (2011). Shortly before use, 0.5 mL of TMB were added to 10 mL of H_2O_2-citrate buffer. The enzyme reaction was stopped with H_2SO_4 (1 mol/L).

2.2. Immunoassay development

2.2.1. Synthesis and characterisation of immunochemicals

PTA was coupled to carrier proteins KLH and GOx in defined molar ratios using the Mannich condensation reaction with formaldehyde as described previously (Abramson et al., 1995). The PTA-KLH conjugate was used for the immunisation of rabbits, whereas the PTA-GOx conjugate was used as solid phase antigen. For preparation of the immunogen, 14 mg KLH were dissolved in 2 mL of aqueous sodium acetate (0.1 mol/L, pH 4.2). Then, 3 mg PTA, dissolved in 0.3 mL of DMSO, and 0.5 mL of 37% formaldehyde solution were added. This mixture was incubated at 37 °C for 21 h, protected from light. For the synthesis of the solid phase antigen (PTA-GOx), 5.4 mg GOx were dissolved in 0.5 mL of aqueous sodium acetate buffer (0.1 mol/L, pH 4.2). To this mixture, 2.5 mg PTA dissolved in 0.25 mL of DMSO and 0.05 mL of 37% formaldehyde solution were added. Then the mixture was incubated for 21 h at 37 °C, protected from light. Both the immunogen and the solid phase

antigen were each dialyzed against 3 × 6 L of PBS pH 7.2 for each at least 6 h. After dialysis, the conjugates were characterised by UV-spectroscopy (200–400 nm). For this purpose, a dilution series of the conjugates was prepared in PBS. Spectra of conjugates were compared with those of the native proteins and of PTA.

2.2.2. Competitive indirect EIA

Custom antibody production was done at Seramun Diagnostica GmbH (Heidesee, Germany). Four rabbits were immunised with the immunogen PTA-KLH conjugate. Each animal was injected intracutaneously with 150 μL (containing approximately 0.5 mg KLH) of the conjugate, mixed with 150 μL PBS and emulsified with 300 μL of Freund's complete adjuvant. Booster injection were done subcutaneously with the same amount and composition of the immunogen, except that Freund's incomplete adjuvant was used. Blood was collected until week 17, and the antibody titers were screened in a competitive indirect enzyme immunoassay using PTA-GOx conjugate as solid phase antigen.

Microtiter plates were coated with PTA-GOx conjugate (3.35 μg/mL in coating buffer pH 9.6, 100 μL per well) and incubated overnight at ambient temperature. Free protein binding sites were blocked with casein sodium salt (2%) in PBS (200 μL per well) for 30 min. The plates were washed and PTA standard solution (10–0.0032 μg/mL, in 10% methanol/PBS, 50 μL per well) and anti-PTA antiserum (1:1000 in PBS, 50 μL per well) were added. The plates were incubated for 1 h at ambient temperature and then washed again, followed by addition of swine anti-rabbit IgG HRP conjugate (1:1000 in PBS, 100 μL per well). The plates were incubated for 1 h, washed again, and then, enzyme substrate/chromogen solution was added (100 μL per well). After 15 min, the enzyme reaction was stopped with 1 mol/L H_2SO_4 (100 μL per well). The UV absorbance values at 450 nm were measured with a model Sunrise microplate reader (Tecan, Crailsheim, Germany) and evaluated by Magellan EIA calculation software (Tecan). For the generation of the standard curve, measured absorbance values (B) for six PTA standard solutions were transformed into relative absorbance values ($B/B_0 \times 100$), setting the absorbance value of the blank solution as B_0.

The detection limit (LOD) of the EIA for PTA was set at 80% relative binding (IC_{20}) of the standard curve. Considering the relatively high non-specific background colour development of the PTA-EIA, corresponding to 30–40% relative binding, a measurement range from 50% to 80% B/B_0 could be utilised for quantification of PTA in fungal mycelium extracts. The cross-reactivity (specificity) of the assay was determined using standard solutions (1 μg/mL) of all available, structurally related indole-diterpene mycotoxins under the conditions of the PTA-EIA. Specificity tests included PAX, verruculogen, terpendole E and lolitrem B.

2.3. Mycotoxin analysis in fungal cultures isolated from food materials

In 2019, food sample material was collected selectively from retail shops and private gardens (walnuts) in Hesse, Germany. Food samples were specifically purchased from retail shops if smaller spots of mould were visible on the surface of a product displayed in the shelf. However, samples were not heavily moulded to an extent, which would have discouraged a customer from purchase, or would have been rejected from regular sale. The intention was to obtain a sufficient variety of mildly mould-infected foods, therefore sample collection was not at all representative for the whole of the food products of a certain retailer.

The sample matrices included dairy products (cheese, desserts; $n = 10$), citrus fruits ($n = 7$) and walnuts ($n = 5$, shell and kernel for each sample). Prior to further analysis, the sample material was incubated at 25 °C for seven days to provoke and enhance fungal growth. Fungal mycelium was wiped off from visibly mouldy spots using a sterile swab. With each swab, one MEA and one SA plate was inoculated (three points). Then the plates were incubated at 25 °C for 5 days. The reference strain *P. crustosum* Thom DSM 62837, after reactivation of the freeze-dried culture in CASO broth according to the instructions of the DSMZ, was also cultured on MEA and SA.

2.4. Characterisation of fungal isolates

A volume of 100 μL of a highly diluted spore solution from three selected fungal cultures (N4, isolated from semi-hard cheese; N11, isolated from lemon; N19, isolated from walnut kernels) was plated onto MG, to obtain single spore colonies. After five days at 25 °C a spore solution from the purified single spore colonies was further transferred to MG and YES medium and then incubated at 25 °C for 10 days in the dark. Then, fungal growth characteristics and colony morphology were evaluated by comparison with published literature (Samson et al., 2002). The three isolates were subjected to further characterisation using molecular identification.

For the isolation of DNA, 100 μL of a suspension made from a single spore culture (10^4 spores/mL of TWS (0.5% Tween 80, 0.85 g/L NaCl)) of each isolate were plated out onto a MG agar plate, covered with a sterile cellophane sheet, and incubated at 25 °C in the dark for 7 days. The plain mycelium was harvested and 150 mg of mycelium was transferred into a 2 mL micro reaction tube. An amount of 700 μL of lysis buffer AP1 (DNeasy Plant Mini Kit Qiagen, Hilden, Germany) was added. A steel ball with a diameter of 5 mm was added along with 7 μL of RNase stock solution (100 mg/mL), according to the suggestions of the manufacturer of the kit. This solution was shredded for 2 × 30 s (6.5 m/s) in a Fast-Prep 24^{TM}5G instrument (MP Biomedical, Eschwege, Germany) and the extract was treated further, exactly as described by the manufacturer of the kit. After various washing steps, the DNA was eluted from the column, by using the respective buffer, and stored at −20 °C until usage.

To characterise the strains at the species level, the internal transcribed spacer (ITS) region and a part of the β-tubulin gene (*benA*) were sequenced. For this purpose, PCR reactions, with primers specific for the ITS region and for a part of the *benA* gene, were performed by using the Taq all-inclusive kit (VWR, Bruchsal, Germany). For the amplification of the ITS region, the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used (White et al., 1990). The primers BT2a (GGTAACCAAATCGGTGCTGCTTTC) and BT2b (ACCCTCAGTGTAGTGACCCTTGGC) were used for the amplification of a part of the *benA* gene (Glass & Donaldson, 1995). A typical reaction mix contained 23.5 μL distilled water, 10 μL enhancer solution, 1 μL dNTP-Mix (10 mmol), 5 μL reaction buffer S, 2.5 μL of each primer (5 pmol/μL), 0.5 μL Taq polymerase and 5 μL template DNA solution. For the amplification of the ITS regions, the following PCR regime was used: i) 95 °C, 3 min; ii) 40 x (95 °C, 0.3 min; 50 °C, 0.4 min; 72 °C, 3 min); iii) 72 °C, 3 min; iv) hold 4 °C, ∞. For the amplification of the part of the *benA* gene, the following scheme was used: i) 94 °C, 1 min; ii) 45 x (94 °C, 1 min; 58 °C, 1 min, 72 °C, 3 min); iii) 72 °C, 3 min; iv) hold 4 °C, ∞. After purification, the PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany). The obtained sequences were compared with the GeneBank database, by using the basic local alignment research tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. EIA analysis of PTA, PAX, and ergoline alkaloids in fungal mycelium agar plug extracts

For mycotoxin extraction, a defined area (1 cm^2) from each agar plate (MEA and SA) showing fungal growth was cut out using a sterile scalpel. The agar plug was mixed with 1 mL of methanol in a 2 mL-Eppendorf vial on a wrist-action shaker (Vortex) for 1 min at high speed, followed by centrifugation at 13,800×*g* for 5 min at 20 °C. The supernatant was collected and stored at 6 °C until analysed. The analysis of fungal mycelium extract samples for PTA was carried out using competitive indirect EIA as previously described in section 2.2.2 The analysis of PAX was performed by a competitive indirect EIA using PAX-*O*-(carboxymethyl) hydroxylamine (PAX-CMO) coupled to bovine serum albumin (BSA) as solid phase antigen (Bauer et al., 2017). Based on

polyclonal antibodies against PAX-CMO-KLH, the PAX-EIA enables highly sensitive detection of PAX with a cross-reactivity to terpendole E of 1.1%. The mean LOD (IC_{20}) of the PAX standard curves performed in our study was 0.7 ng/mL, which is largely in agreement with a value of 0.47 ng/mL reported by Bauer et al. (2017).

For the detection of ergoline alkaloids, a competitive direct EIA using highly sensitive polyclonal antibodies against ergometrine-KLH was used, with a standard curve LOD (ergometrine) of 0.02 ng/mL (Gross et al., 2018). Although this EIA is most sensitive to ergometrine, it also detects all other ergot alkaloids in a very low concentration range (ng/mL). Cross-reactivity relative to ergometrine was in the range of 0.01–7% for all other ergot alkaloids, with ergotamine showing the strongest cross-reactivity with 7%. With regard to isofumigaclavine A, the cross-reactivity of the test system was about 60% (Sinner, 2017). The ergometrine EIA is basically suitable for a group-specific detection of ergoline alkaloids in fungal extracts. Results were expressed as ergometrine equivalents.

For EIA analysis, the mycelium extracts were diluted at least 1:10 in PBS pH 7.2 for PTA-EIA, 1:5 in PBS pH 7.2 for the PAX-EIA and 1:10 in PBS pH 6.0 for the ergoline alkaloid EIA. Further dilutions of the mycelium extracts and dilutions of the corresponding standard solution were made if necessary, using 10% methanol/PBS pH 7.2 for the PTA-EIA, 20% methanol/PBS pH 7.2 for the PAX-EIA, and 5% acetonitrile/PBS pH 6.0 for the ergoline alkaloid EIA. Taking into account the minimum fungal extract dilution factors as listed above, the LOD in mycelium extracts was 2 μg/mL for PTA, 0.004 μg/mL for PAX and 0.0002 μg/mL for ergoline alkaloids (ergometrine equivalents). Four replicate wells were tested for each solution of toxin standard and sample extract.

2.6. Confirmatory analysis of PTA and PAX by HPLC-MS

HPLC−MS/MS analysis of fungal mycelium extract samples was carried out on a 1260 Infinity LC system from Agilent (Waldbronn, Germany) coupled to a QTrap 5500 mass spectrometer from SCIEX (Darmstadt, Germany) using the method of Kalinina et al. (2018) but with additional selected reaction monitoring transitions for PAX and DesoxyPAX. The following parameters for dwell time (DT), declustering potential (DP) and collision energy voltage (CE) were set: PAX quantifier *m/z* 436.2/182.1, DT 20 ms, DP 200 V, CE 45 V; PAX qualifier 1: *m/z* 436.2/130.1, DT 20 ms, DP 200 V, CE 50 V; PAX qualifier 2: *m/z* 436.2/167.1, DT 20 ms, DP 220 V, CE 92 V. DesoxyPAX quantifier: *m/z* 420.2/182.2, DT 20 ms, DP 220 V, CE 45; DesoxyPAX qualifier: *m/z* 420.2/130.1, DT 20 ms, DP 220 V, CE 47 V.

Sample preparation for the analysis of PTA and PAX in fungal mycelium extracts was performed according to Bauer et al. (2017). Prior to injection, the samples were either diluted by a factor of five with methanol water (7/3, v/v) (N4, N11) or evaporated to dryness at 40 °C under a stream of nitrogen and redissolved in 100 μL acetonitrile-water (2/8, v/v) (N19, N25). After 5 min of sonification, samples were centrifuged (2000×*g*, 5 min), and 20 μL of the supernatant were injected into the HPLC-MS/MS system. For comparison, combined PAX and PTA standards at concentrations of 1, 2.5, 5, 10, 25, 50 and 100 ng/mL were prepared by dilution from a stock solution containing 1 mg/mL PAX in methanol and aliquots treated like the samples above. For PAX and PTA, the LOD was 2.2 ng/mL and the LOQ was 7.2 ng/mL. Qualitative references for Penitrem B–F (PTB-PTF) were available from the study by Kalinina et al. (2018). PAX eluted at 7.88 min, PTA at 8.17 min, PTB at 9.14 min, PTC at 9.51 min, PTD at 8.95 min, PTE at 7.58 min, and PTF at 9.66 min. Potential 13-desoxy-PAX was assigned to a retention time of 8.68 min.

3. Results and discussion

3.1. Development of the PTA-EIA

The Mannich condensation reaction using formaldehyde (Mannich & Krösche, 1912) made it possible to couple PTA to carrier proteins KLH and GOx, so that stable hapten-protein-complexes could be produced. PTA is a complex molecule containing three hydroxyl and one indole group, all of which may react with formaldehyde. Although it was not possible to determine the exact conjugation site of PTA to the carrier proteins, we assume that the indole nitrogen was the preferable position for formaldehyde condensation. UV-spectroscopy of the PTA-BSA and the PTA-KLH conjugates showed increased absorbance at wavelengths corresponding to the spectrum of PTA standard toxin, most notably at 235 nm. This qualitatively indicated successful conjugation of PTA to carrier proteins. In indirect EIA, PTA specific antibody titers could be detected in three out of four rabbits immunised with PTA-KLH, with maximum titers of about 1:10,000, indicating a moderate immunogenicity of the KLH conjugate. Binding inhibition by free PTA in competitive indirect EIA revealed that even the best antiserum was only moderately sensitive, with a 50% inhibition dose at 3.0 μg/mL. The standard curve enabled detection of PTA in the ng/mL range, but had a relatively high background absorbance of about 45% B/B_0 (Fig. 1). Therefore, the dynamic measuring range was restricted to a quasilinear range between 50% (IC_{50}) and 80% (IC_{20}) of the blank value, corresponding to about 10,000–80 ng/mL. The mean ($n = 7$) PTA standard curve parameters IC_{50} and IC_{20} were at 3.1 ± 1.4 μg/mL and 0.22 ± 0.17 μg/mL, the latter set as the detection limit. Penitrems B to F were not available for specificity testing, but considering the high degree of structural similarity with PTA, it appears to be very likely that at least some of these compounds could be detected in the test system as well. Broad specificity for the penitrem group of the PTA-EIA would be very advantageous for food analysis, and should be tested as soon as toxin standards for these penitrems become available. The test system showed no competitive binding inhibition by other indole-diterpenes such as PAX, verruculogen, terpendole E, and lolitrem B, each at a level of 1 μg/mL. This was not unexpected because of the major structural differences between these compounds and PTA.

3.2. Toxigenic fungi from mouldy foods and mycotoxin production in culture

With a mean standard curve detection limit of 0.22 μg/mL, the PTA-EIA was not sufficiently sensitive to directly analyse PTA in diluted extracts of food sample material at levels, which have been reported to occur under natural conditions. For example, Young et al. (2003) have reported PTA levels of 20–50 μg/g in cheese, which had caused intoxications in dogs. More recently, PTA was reported to occur in about 10% of cheese samples from the European single market, although at relatively low average and maximum concentrations of 28.4 μg/kg and 429 μg/kg, respectively (Kalinina et al., 2018). We therefore sought to investigate the PTA-producing capability of fungal material obtained from slightly mould-contaminated foods, as a first application of the test system. To cover a wider spectrum of indole-type toxins, two other EIAs, which had been reported earlier, were included in these experiments. One was an EIA for PAX (Bauer et al., 2017), which is a structurally different indole-diterpene tremorgen, and a generic EIA for the ergoline class of mycotoxins (Gross et al., 2018). The ergoline EIA has a broad specificity not only for ergot alkaloids, but also detects mycotoxins of the fumigaclavine and the isofumigaclavine type (Sinner, 2017). Both the PAX-EIA and the ergoline EIA have been applied previously to study the toxin profiles of endophytic fungi in grass and grass seeds (Bauer et al., 2018).

Since the food samples under study showed just small mouldy spots on the day of sample collection, which was not enough to harvest mycelium for further analysis, further fungal growth was promoted by incubation at 25 °C for one week. Then, all samples yielded sufficient greenish or grayish fungal material for transfer onto MEA and SA. According to colony morphology, most moulds could be identified as *Penicillium* species. While fungal material obtained from each sample of cheese and citrus fruit had uniform macroscopic appearance, fungal

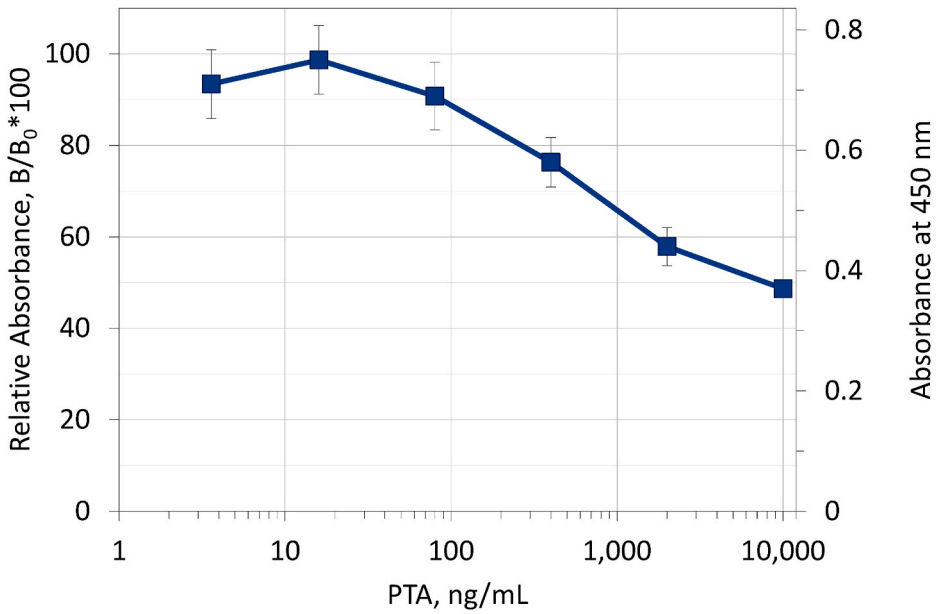

Fig. 1. Typical standard curve of the competitive indirect EIA for PTA. Each of the six standard curve data points represent the mean absorbance of four replicate wells, the coefficient of variation ranged from 2 to 10%. The absorbance value of the blank wells (B_0) was typically in the range of 0.7 and 1.3 units.

material from walnuts (fruit or shell) consistently grew as mixed cultures, and both *Penicillium* and *Aspergillus* type structures were found to be dominant according to microscopic evaluation.

Analyses of mycelium extracts of fungal cultures from MEA and SA by the three EIAs yielded positive results for 19 out of 27 isolates in at least one test system (Table 1). Although the highest PTA concentration was found for an extract from MEA, levels in extracts from both media were not consistently higher in MEA. Overall, extracts from eight samples were positive for PTA, 16 were positive for PAX, and nine were positive in the ergoline EIA. However, the levels of PTA in each one sample of cheese, lemon, and walnut kernel by far exceeded those, which were found by the other two EIAs.

Half of the isolates from cheese were positive for PTA, especially those obtained from semi-hard cheeses. For cheese isolates, the highest concentration of PTA (90 μg/mL) was produced by isolate N4, obtained from a semi-hard cheese with (non-specified) herbs. Cheese is a good substrate for growth of *Penicillium* species (Bullerman, 1981; Lund et al., 1995), including PTA-producing species (Kalinina et al., 2018; Richard & Arp, 1979; Young et al., 2003). It seems possible that the original source of fungi was from herbs. Overall, the toxin-producing potential of isolates from cheese seems to be in a range, which has to be considered as relevant for acute toxicity. *P. crustosum* and/or PTA in cheese has been associated with cases of tremorgenic poisoning in dogs (Richard & Arp, 1979; Young et al., 2003).

A high number of extracts were also positive in the PAX-EIA ($n = 16$) and in the EIA for ergoline alkaloids ($n = 9$), albeit at lower levels of

Table 1
EIA results for indole-alkaloids in mycelium extracts of fungal cultures isolated from various foods. Only isolates, which yielded a positive result at least in one EIA system are listed. Each isolate was tested after cultivation on malt extract agar (MEA) and on Sabouraud glucose chloramphenicol selective agar (SA). For each mycelium extract a single determination was performed. HPLC/MS-MS results for four extracts, selected according to their EIA results, are shown for comparison.

Isolate	Source	PTA, μg/mL				PAX, μg/mL				Ergoline alkaloids*, μg/mL	
		EIA		LC-MS/MS		EIA		LC-MS/MS		EIA	
		MEA	SA	MEA	SA	MEA	SA	MEA	SA	MEA	SA
N1	Hard cheese, grated	<2	3	–	–	0.009	0.005	–	–	<0.0002	<0.0002
N2	Hard cheese, grated	<2	<2	–	–	0.02	<0.004	–	–	0.1	0.0008
N3	Semi-hard cheese	<2	<2	–	–	0.008	<0.004	–	–	0.004	0.05
N4[1]	Semi-hard cheese with herbs	90	60	0.15	–	0.2	0.2	n.d.	–	<0.0002	0.0003
N5	Semi-hard cheese	<2	<2	–	–	<0.004	<0.004	–	–	0.002	0.0002
N6	Semi-hard cheese with herbs	<2	7	–	–	<0.004	<0.004	–	–	0.3	0.3
N7	Semi-hard cheese	4	<2	–	–	<0.004	0.004	–	–	0.3	0.3
N8	Cream cheese with herbs	<2	<2	–	–	0.004	0.005	–	–	0.2	0.2
N9	Creme Fraiche	<2	<2	–	–	0.02	<0.004	–	–	0.2	0.001
N10	Mousse au Chocolat	<2	<2	–	–	<0.004	0.005	–	–	<0.0002	<0.0002
N11[2]	Lemon	200	30	1	–	2	3	0.2	–	<0.0002	<0.0002
N15	Orange	<2	2	–	–	<0.004	<0.004	–	–	<0.0002	<0.0002
N17	Pomelo	<2	<2	–	–	<0.004	0.005	–	–	<0.0002	<0.0002
N18	Walnut kernel	<2	<2	–	–	0.2	0.007	–	–	<0.0002	<0.0002
N19[2]	Walnut kernel	70	90	5	–	4	4	0.3	–	<0.0002	<0.0002
N20	Walnut kernel	<2	<2	–	–	<0.004	0.02	–	–	<0.0002	<0.0002
N21	Walnut kernel	<2	<2	–	–	0.02	0.006	–	–	<0.0002	<0.0002
N22	Walnut kernel	<2	<2	–	–	<0.004	0.004	–	–	<0.0002	0.4
N25	Walnut shell	<2	6	–	0.15	<0.004	0.3	–	n.d.	<0.0002	<0.0002
N28	Reference strain (*P. crustosum*)	11	2	–	–	0.01	<0.004	–	–	0.002	0.002

-, not analysed; n.d., not detected; * expressed as ergometrine equivalents; 1 isolate identified as *P. polonicum*; 2, isolate identified as *P. crustosum*.

0.0002–4 μg/mL. Considering the reactivity pattern of the latter two EIAs (Bauer et al., 2017; Gross et al., 2018; Sinner, 2017), these results are mostly qualitative in nature, because the identity of the toxins was not known. It is reasonable to assume that for ergolines, isofumigaclavine A, which has about 60% cross-reactivity in the ergoline alkaloid EIA (Sinner, 2017) could have been the cause of positive results. The production of isofumigaclavine A is commonly consistent with the mycotoxin profile of *P. roqueforti*, which - except of its intended use in blue-veined cheese - is considered a typical spoilage fungus of dairy products, and has already been isolated from hard, semi-hard, and semi-soft cheeses (Hocking & Faedo, 1992; Kure et al., 2001; Lund et al., 1995; Moreau & Moss, 1979; Scott et al., 1976). Overall, the results obtained by the three EIA methods clearly indicate that cheese after spoilage may be contaminated with a highly diverse mixture of different indole alkaloid mycotoxins.

One isolate from lemon (N11), after cultured on MEA, was strongly positive in the EIAs for PTA (200 μg/mL) and PAX (2 μg/mL), while no ergoline alkaloids could be detected. Few data are available on the presence of *P. crustosum* in citrus fruits (Garcha & Singh, 1976). To the best of our knowledge, this is the first report of isolation of PTA-producing *P. crustosum* from citrus fruits. One isolate from walnut (N19) yielded highly positive results in the PTA-EIA (70–90 μg/mL) and the PAX-EIA (4 μg/mL), and again was negative for ergoline alkaloids. The isolation of PTA-producing *P. crustosum* from walnuts and chestnuts has been described in previous studies, some in the context of food poisoning (Botha et al., 2019; Overy et al., 2003; Richard et al., 1981). The *P. crustosum* strain Thom DSM 62837, used as PTA-positive control, was positive in the PTA-EIA at a level of approximately 11 μg/mL on MEA, and at 2 μg/mL on SA. Additionally, this strain was weakly positive in the EIAs for PAX and ergoline alkaloids. Based on the results of the positive control and taking into account the biosynthesis of the indole-diterpenes, the co-occurrence of PAX and PTA was not unexpected since PAX can be a precursor of the penitrem synthesis (Mantle & Penn, 1989).

3.3. Control analyses

HPLC-MS/MS was used as a control method for PTA and PAX in four selected mycelium extracts (N4; N11; N19; N25). SRM chromatograms of sample N11 are shown in Fig. 2. All four mycelium extracts showed signals for PTA whereas PAX could only be detected in mycelium extracts of N11 and N19. Furthermore, a signal with the characteristic *m/z* value and fragmentation pattern of 13-desoxy-PAX could be detected in all mycelium extracts that were positive in the PAX-EIA, indicating that the PAX-EIA most likely cross-reacts with this derivative to a considerable extent as previously observed by Bauer et al. (2017). However, quantification and unequivocal identification of this PAX analogue was not possible by HPLC-MS/MS due to the lack of reference standards. In all extracts, signals were also detected that could be assigned to the penitrems B–F, but again the lack of standards prohibited quantitation. However, the presence of penitrem analogues in these extracts provides a plausible explanation for the marked quantitative discrepancies between EIA results and HPLC-MS/MS results for PTA and PAX. It is a significant limitation of our study that as of yet we could not quantify the

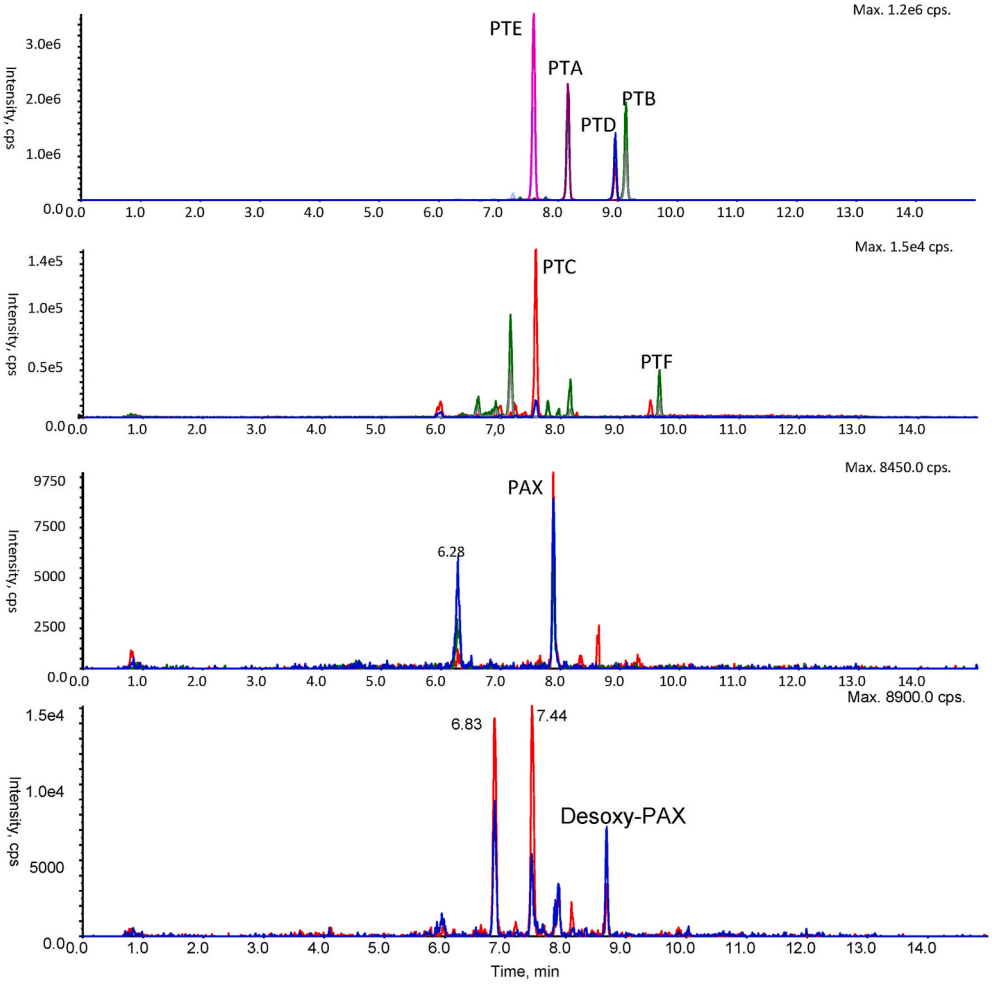

Fig. 2. HPLC-MS/MS chromatogram of sample N11 showing the different SRM traces for the analytes PTA-PTF, PAX and Desoxy-PAX. Additional signals with the characteristic SMR transitions of PAX and 13-Desoxy-PAX are present at retention times of 6.28, 6.83 and 7.44 min.

concentration of total penitrems or total paxillines in the fungal extracts. The EIAs may therefore strongly overestimate the total toxin content for both parent toxins plus analogues. On the other hand, the results of HPLC-MS/MS (Fig. 2) indicate that total penitrem content in mycelium extracts was dominated by penitrems other than PTA, indicating the potential of the EIA for group-specificity. This can be interpreted as a benefit of the EIAs, if it could indicate the presence of toxins, which cannot be quantified yet. This may also explain the failure of previous studies to assign plausible mycotoxin levels to cases of intoxication (Botha et al., 2019; Richard et al., 1981). Further work should therefore aim at elucidation of the total toxin profiles of an extended number of *Penicillium* strains, using EIA methods for pre-screening of fungal extracts. Another remarkable finding of this study is that for the first time, *P. polonicum* could be identified as a PTA producer.

In order to further characterise the results obtained by the EIAs, the three isolates for which the highest PTA levels had been found in methanolic extracts (N4; N11; N19), had been selected for further identification. Sequencing of the ITS region and partial sequencing of the β-tubulin gene (Visagie et al., 2014) clearly identified isolate N4 (from cheese with herbs) as *P. polonicum*, whereas isolates N11 (citrus) and N19 (walnut) were both identified as *P. crustosum* (Table 1). This was in agreement with colony morphology on MG and MEA. On MG, the two *P. crustosum* strains showed a crustose colony surface with a grey-green to pale green colour, whereas *P. polonicum* produced blue-green to blue conidia, with an intense yellow reverse of the colony. On YES medium, both species showed heavy sporulation. *P. polonicum* had a distinct yellow colour of the reverse side of the colony (Samson et al., 2002). Although *P. polonicum* so far is considered to be a non-typical spoilage agent of dairy products, it has previously been found in grana cheese (Decontardi et al., 2018). While *P. crustosum* is a well-known producer of PTA, this study reports for the first time that *P. polonicum* also produces PTA and analogues. This adds to previous results, which showed that certain strains of *P. polonicum* were able to produce the polyketide mycotoxins citrinin (Chen et al., 2020) and verrucosidin (Prencipe et al., 2018). *P. polonicum* may therefore have greater importance as a multi-mycotoxin producer and a food poisoning agent than currently known.

3.4. Conclusions

This study is the first one describing the production of antibodies against PTA and their use in EIA for PTA analysis in extracts of food spoilage fungi after culture. Although the lack of a broader set of toxin standards currently prevents a clear quantification, comparison with LC-MS/MS suggests that the EIA detects both PTA and structural analogues. The finding that fungi from food spoilage produce PTA, PAX, their analogues, plus other ergoline alkaloids in some cases, indicates that a greater focus should be directed on indole alkaloids as a cause of food poisoning of humans and pet animals. Further work aims on improving the sensitivity of the PTA, to enable direct detection of this toxin in diluted food extracts.

Funding

This study was financially supported by the Brigitte and Wolfram Gedek Foundation (Ismaning, Germany).

CRediT authorship contribution statement

Christina Rehagel: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Ömer Akineden:** Investigation, Supervision, Writing – review & editing. **Rolf Geisen:** Investigation, Writing – review & editing. **Benedikt Cramer:** Investigation, Writing – review & editing. **Madeleine Plötz:** Funding acquisition, Writing – review & editing. **Ewald Usleber:** Funding acquisition, Resources, Supervision, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank the Brigitte and Wolfram Gedek-Foundation (Ismaning, Germany) for financial support.

References

Abramson, D., Usleber, E., & Märtlbauer, E. (1995). An indirect enzyme immunoassay for the mycotoxin citrinin. *Applied and Environmental Microbiology, 61*, 2007–2009. https://doi.org/10.1128/aem.61.5.2007-2009.1995

Ackermann, Y., Curtui, V., Dietrich, R., Gross, M., Latif, H., Märtlbauer, E., & Usleber, E. (2011). Widespread occurrence of low levels of alternariol in apple and tomato products, as determined by comparative immunochemical assessment using monoclonal and polyclonal antibodies. *Journal of Agricultural and Food Chemistry, 59*, 6360–6368. https://doi.org/10.1021/jf201516f

Bauer, J. I., Gross, M., Cramer, B., Humpf, H.-U., Hamscher, G., & Usleber, E. (2018). Immunochemical analysis of paxilline and ergot alkaloid mycotoxins in grass seeds and plants. *Journal of Agricultural and Food Chemistry, 66*, 315–322. https://doi.org/10.1021/acs.jafc.7b05580

Bauer, J. I., Gross, M., Cramer, B., Wegner, S., Hausmann, H., Hamscher, G., & Usleber, E. (2017). Detection of the tremorgenic mycotoxin paxilline and its desoxy analog in ergot of rye and barley: A new class of mycotoxins added to an old problem. *Analytical and Bioanalytical Chemistry, 409*, 5101–5112. https://doi.org/10.1007/s00216-017-0455-y

Botha, C. J., Visagie, C. M., & Sulyok, M. (2019). Putative neuromycotoxicoses in an adult male following ingestion of moldy walnuts. *Mycotoxin Research, 35*, 9–16. https://doi.org/10.1007/s12550-018-0326-1

Boysen, M., Skouboe, P., Frisvad, J. C., & Rossen, L. (1996). Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. *Microbiology, 142*, 541–549. https://doi.org/10.1099/13500872-142-3-541

Braselton, W. E., & Johnson, M. J. (2003). Thin layer chromatography convulsant screen extended by gas chromatography-mass spectrometry. *Journal of Veterinary Diagnostic Investigation, 15*, 42–45. https://doi.org/10.1177/104063870301500109

Braselton, W. E., & Rumler, P. C. (1996). MS/MS screen for the tremorgenic mycotoxins roquefortine and penitrem A. *Journal of Veterinary Diagnostic Investigation, 8*, 515–518. https://doi.org/10.1177/104063879600800427

Bullerman, L. B. (1981). Public health significance of molds and mycotoxins in fermented dairy products. *Journal of Dairy Science, 64*, 2439–2452. https://doi.org/10.3168/jds.S0022-0302(81)82869-X

Cavanagh, J. B., Holton, J. L., Nolan, C. C., Ray, D. E., Naik, J. T., & Mantle, P. G. (1998). The effects of the tremorgenic mycotoxin penitrem A on the rat cerebellum. *Veterinary Pathology, 35*, 53–63. https://doi.org/10.1177/030098589803500105

Chen, L., Guo, W., Zheng, Y., Zhou, J., Liu, T., Chen, W., Liang, D., Zhao, M., Zhu, Y., Wu, Q., & Zhang, J. (2020). Occurrence and characterization of fungi and mycotoxins in contaminated medicinal herbs. *Toxins, 12*(1), 30. https://doi.org/10.3390/toxins12010030

Cole, R. J., Dorner, J. W., Cox, R. H., & Raymond, L. W. (1983). Two classes of alkaloid mycotoxins produced by *Penicillium crustosum* Thom isolated from contaminated beer. *Journal of Agricultural and Food Chemistry, 31*, 655–657. https://doi.org/10.1021/jf00117a045

De Jesus, A. E., Gorst-Allman, C. P., Steyn, P. S., van Heerden, F. R., Vleggaar, R., Wessels, P. L., & Hull, W. E. (1983c). Tremorgenic mycotoxins from *Penicillium crustosum*. Biosynthesis of penitrem A. *Journal of the Chemical Society Perkin Transactions, 1*, 1863–1868. https://doi.org/10.1039/P19830001863

De Jesus, A. E., Steyn, P. S., van Heerden, F. R., Vleggaar, R., Wessels, P. L., & Hull, W. E. (1981). Structure and biosynthesis of the penitrems A–F, six novel tremorgenic mycotoxins from *Penicillium crustosum*. *Journal of the Chemical Society Chemical Communications, 6*, 289–291. https://doi.org/10.1039/C39810000289

De Jesus, A. E., Steyn, P. S., van Heerden, F. R., Vleggaar, R., Wessels, P. L., & Hull, W. E. (1983a). Tremorgenic mycotoxins from *Penicillium crustosum*: Isolation of penitrems A-F and the structure elucidation and absolute configuration of penitrem A. *Journal of the Chemical Society Perkin Transactions, 1*, 1847–1856. https://doi.org/10.1039/P19830001847

De Jesus, A. E., Steyn, P. S., van Heerden, F. R., Vleggaar, R., Wessels, P. L., & Hull, W. E. (1983b). Tremorgenic mycotoxins from *Penicillium crustosum*. Structure elucidation and absolute configuration of penitrems B-F. *Journal of the Chemical Society Perkin Transactions, 1*, 1857–1861.

Decontardi, S., Soares, C., Lima, N., & Battilani, P. (2018). Polyphasic identification of Penicillia and Aspergilli isolated from Italian grana cheese. *Food Microbiology, 73*, 137–149. https://doi.org/10.1016/j.fm.2018.01.012

Eriksen, G. S., Jäderlund, K. H., Moldes-Anaya, A., Schönheit, J., Bernhoft, A., Jaeger, G., Rundberget, T., & Skaar, I. (2010). Poisoning of dogs with tremorgenic *Penicillium*

toxins. *Medical Mycology, 48*, 188–196. https://doi.org/10.3109/13693780903225821

Garcha, H. S., & Singh, V. (1976). *Penicillium crustosum*, a new pathogen of *Citrus Reticulata* (Mandarin) from India. *Plant Disease Reporter, 60*, 252–254.

Glass, N. L., & Donaldson, G. C. (1995). Development of premier sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology, 61*, 1323–1330.

González, M. C., Lull, C., Moya, P., Ayala, I., Primo, J., & Primo Yúfera, E. (2003). Insecticidal activity of penitrems, including penitrem G, a new member of the family isolated from *Penicillium crustosum*. *Journal of Agricultural and Food Chemistry, 51*, 2156–2160. https://doi.org/10.1021/jf020983e

Gordon, K. E., Masotti, R. E., & Waddell, W. R. (1993). Tremorgenic encephalopathy: A role of mycotoxins in the production of CNS disease in humans? *Canadian Journal of Neurological Sciences, 20*, 237–239. https://doi.org/10.1017/S0317167100048010

Gross, M., Curtui, V., & Usleber, E. (2018). Detection of total ergot alkaloids in cereal flour and in bread by a generic enzyme immunoassay method. *Journal of AOAC International, 101*, 618–626. https://doi.org/10.5740/jaoacint.17-0346

Hayashi, H., Asabu, Y., Murao, S., Nakayama, M., & Arai, M. (1993). A new congener of penitrems, 6-bromopenitrem E, from *Penicillium simplicissimum* AK-40. *Chemistry Express, 8*, 233–236.

Hayes, A. W., Presley, D. B., & Neville, J. A. (1976). Acute toxicity of penitrem A in dogs. *Toxicology and Applied Pharmacology, 35*, 311–320. https://doi.org/10.1016/0041-008X(76)90290-8

Hocking, A. D., & Faedo, M. (1992). Fungi causing thread mould spoilage of vacuum packaged Cheddar cheese during maturation. *International Journal of Food Microbiology, 16*, 123–130. https://doi.org/10.1016/0168-1605(92)90005-N

Hocking, A. D., Holds, K., & Tobin, N. F. (1988). Intoxication by tremorgenic mycotoxin (penitrem A) in a dog. *Australian Veterinary Journal, 65*, 82–85. https://doi.org/10.1111/j.1751-0813.1988.tb07366.x

Hou, C. T., Ciegler, A., & Hesseltine, C. W. (1971). Tremorgenic toxins from penicillia. III. Tremortin production by *Penicillium* species on various agricultural commodities. *Applied Microbiology, 21*, 1101–1103.

Kalinina, S. A., Jagels, A., Hickert, S., Mauriz Marques, L. M., Cramer, B., & Humpf, H.-U. (2018). Detection of the cytotoxic penitrems A−F in cheese from the European single market by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 66*, 1264–1269. https://doi.org/10.1021/acs.jafc.7b06001

Knaus, H. G., McManus, O. B., Lee, S. H., Schmalhofer, W. A., Garcia-Calvo, M., Helms, L. M., Sanchez, M., Giangiacomo, K., & Reuben, J. P. (1994). Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochemistry, 33*, 5819–5828. https://doi.org/10.1021/bi00185a021

Kozák, L., Szilágyi, Z., Tóth, L., Pócsi, I., & Molnár, I. (2019). Tremorgenic and neurotoxic paspaline-derived indole-diterpenes: Biosynthetic diversity, threats and applications. *Applied Microbiology and Biotechnology, 103*, 1599–1616. https://doi.org/10.1007/s00253-018-09594-x

Kure, C. F., Wasteson, Y., Brendehaug, J., & Skaar, I. (2001). Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. *International Journal of Food Microbiology, 70*, 21–27. https://doi.org/10.1016/S0168-1605(01)00520-7

Lewis, P. R., Donoghue, M. B., Hocking, A. D., Cook, L., & Granger, L. V. (2005). Tremor syndrome associated with a fungal toxin: Sequelae of food contamination. *Medical Journal of Australia, 182*, 582–584. https://doi.org/10.5694/j.1326-5377.2005.tb06819.x

Lund, F., Filtenborg, O., & Frisvad, J. C. (1995). Associated mycoflora of cheese. *Food Microbiology, 12*, 173–180. https://doi.org/10.1016/S0740-0020(95)80094-8

Mannich, C., & Krösche, W. (1912). Über ein Kondensationsprodukt aus Formaldehyd, Ammoniak und Antipyrin. *Archiv der Pharmazie, 250*(1), 647–667. https://doi.org/10.1002/ardp.19122500151

Mantle, P. G., & Penn, J. (1989). A role for paxilline in the biosynthesis of indole-diterpenoid penitrem mycotoxins. *Journal of the Chemical Society Perkin Transactions, 1*, 1539–1540. https://doi.org/10.1039/P19890001539

Mantle, P. G., & Penny, R. H. C. (1981). Tremorgenic mycotoxins and neurological disorders-a review. In C. G, S. Grunsell, & F. W. G. Hill (Eds.), *The Veterinary Annual 21st* (pp. 51–62). Bristol: John Wright & Sons.

Moldes-Anaya, A., Rundberget, T., Fæste, C. K., Eriksen, G. S., & Bernhoft, A. (2012). Neurotoxicity of *Penicillium crustosum* secondary metabolites: Tremorgenic activity of orally administered penitrem A and thomitrem A and E in mice. *Toxicon, 60*, 1428–1435. https://doi.org/10.1016/j.toxicon.2012.10.007

Moreau, C., & Moss, M. (1979). Diverse *Penicillium* toxicoses. In C. Moreaux, & M. Moss (Eds.), *Moulds, toxins and food* (pp. 204–210). Chichester, New York, Brisbane, Toronto: John Wiley and Sons.

Munday, J. S., Thompson, D., Finch, S. C., Babu, J. V., Wilkins, A. L., Menna, M. E., & Miles, C. O. (2008). Presumptive tremorgenic mycotoxicosis in a dog in New Zealand, after eating mouldy walnuts. *New Zealand Veterinary Journal, 56*(3), 145–147. https://doi.org/10.1080/00480169.2008.36823

Naudé, T. W., O'Brien, O. M., Rundberget, T., McGregor, A. D. G., Roux, C., & Flåøyen, A. (2002). Tremorgenic neuromycotoxicosis in 2 dogs ascribed to the ingestion of penitrem A and possibly roquefortine in rice contaminated with *Penicillium crustosum*: Clinical communication. *Journal of the South African Veterinary Association, 73*, 211–215. https://doi.org/10.4102/jsava.v73i4.589

Norris, P. J., Smith, C. C. T., De Belleroche, J., Bradford, H. F., Mantle, P. G., Thomas, A. J., & Penny, R. H. C. (1980). Actions of tremorgenic fungal toxins on neurotransmitter release. *Journal of Neurochemistry, 34*, 33–42. https://doi.org/10.1111/j.1471-4159.1980.tb04618.x

Overy, D. P., Seifert, K. A., Savard, M. E., & Frisvad, J. C.((2003). Spoilage fungi and their mycotoxins in commercially marketed chestnuts. *International Journal of Food Microbiology, 88*, 69–77. https://doi.org/10.1016/s0168-1605(03)00086-2

Prencipe, S., Siciliano, I., Gatti, C., Garibaldi, A., Gullino, M. L., Botta, R., & Spadaro, D. (2018). Several species of *Penicillium* isolated from chestnut flour processing are pathogenic on fresh chestnuts and produce mycotoxins. *Food Microbiology, 76*, 396–404. https://doi.org/10.1016/j.fm.2018.07.003

Reddy, P., Guthridge, K., Vassiliadis, S., Hemsworth, J., Hettiarachichige, I., Spangenberg, G., & Rochfort, S. (2019). Tremorgenic mycotoxins: Structure diversity and biological activity. *Toxins, 11*, 302. https://doi.org/10.3390/toxins11050302

Richard, J. L., & Arp, L. H. (1979). Natural occurrence of the mycotoxin penitrem A in moldy cream cheese. *Mycopathologia, 67*, 107–109. https://doi.org/10.1007/bf00440681

Richard, J. L., Bacchetti, P., & Arp, L. H. (1981). Moldy walnut toxicosis in a dog, caused by the mycotoxin, penitrem A. *Mycopathologia, 76*, 55–58. https://doi.org/10.1007/bf00761899

Rundberget, T., Skaar, I., & Flåøyen, A. (2004). The presence of *Penicillium* and *Penicillium* mycotoxins in food wastes. *International Journal of Food Microbiology, 90*, 181–188. https://doi.org/10.1016/s0168-1605(03)00291-5

Rundberget, T., Skaar, I., O'Brien, O., & Flåøyen, A. (2004). Penitrem and thomitrem formation by *Penicillium crustosum*. *Mycopathologia, 157*, 349–357. https://doi.org/10.1023/b:myco.0000024180.99262.b1

Sallam, A. A., Houssen, W. E., Gissendanner, C. R., Orabi, K. Y., Foudah, A. I., & El Sayed, K. A. (2013). Bioguided discovery and pharmacophore modeling of the mycotoxic indole diterpene alkaloids penitrems as breast cancer proliferation, migration, and invasion inhibitors. *Medicinal Chemistry Communication, 4*, 1360–1369. https://doi.org/10.1039/C3MD00198A

Samson, R. A., Hoekstra, E. S., Frisvad, J. C., & Filtenborg, O. (2002). *Introduction to food- and airborne fungi*. Utrecht, The Netherlands: Centraalbureau voor Schimmelcultures, ISBN 90-70351-42-0.

Santini, A., Mikušová, P., Sulyok, M., Krska, R., Labuda, R., & Šrobárová, A. (2014). *Penicillium* strains isolated from Slovak grape berries taxonomy assessment by secondary metabolite profile. *Mycotoxin Research, 30*, 213–220. https://doi.org/10.1007/s12550-014-0205-3

Scott, P. M., Merrien, M. A., & Polonsky, J. (1976). Roquefortine and isofumigaclavine A, alkaloids from *Penicillium roqueforti*. *Experientia, 32*, 140–142.

Sinner, N. (2017). *Detection of isofumigaclavine A-mycotoxin in blue moldy cheese*. PhD thesis, Dairy Sciences. Institute of Veterinary Food Science, Justus-Liebig-University Giessen.

Ueno, Y., & Ueno, I. (1978). Toxicology and biochemistry of mycotoxins. In K. Uraguchi, & M. Yamazaki (Eds.), *Toxicology, biochemistry and pathology of mycotoxins* (pp. 107–188). New York: John Willey & Sons.

Uhlig, S., Ivanova, L., Voorspoels, P., & Fæste, C. K. (2020). In vitro toxicokinetics and phase I biotransformation of the mycotoxin penitrem A in dogs. *Toxins, 12*, 293. https://doi.org/10.3390/toxins12050293

Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S. B., Klaassen, C. H. W., Perrone, G., Seifert, K. A., Varga, J., Yaguchi, T., & Samson, R. A. (2014). Identification and nomenclature of the genus *Penicillium*. *Studies in Mycology, 78*, 343–371. https://doi.org/10.1016/j.simyco.2014.09.001

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols. A guide the methods and applications* (pp. 315–322). New York: Academic Press.

Wilson, B. J., Hoekman, T., & Dettbarn, W. D. (1972). Effects of a fungus tremorgenic toxin (penitrem A) on transmission in rat phrenic nerve-diaphram preparations. *Brain Research, 40*, 540–544. https://doi.org/10.1016/0006-8993(72)90159-X

Wilson, B. J., Wilson, C. H., & Hayes, A. W. (1968). Tremorgenic toxin from *Penicillium cyclopium* grown on food materials. *Nature, 220*, 77–78. https://doi.org/10.1038/220077b0

Young, K. L., Villar, D., Carson, T. L., Imerman, P. M., Moore, R. A., & Bottoff, M. R. (2003). Tremorgenic mycotoxin intoxication with penitrem A and roquefortine in two dogs. *Journal of the American Veterinary Medical Association, 222*, 52–53. https://doi.org/10.2460/javma.2003.222.52

6.2 Microbiological and mycotoxicological analyses of processed cereal-based complementary foods for infants and young children from the German market

Rehagel C, Akineden Ö, Usleber E

Journal of Food Science 87, 1810-1822 (2022)

Author contributions:

Rehagel C: Conceptualization, formal analysis, investigation, validation, visualization, writing - original draft; writing - review & editing. **Akineden Ö**: Conceptualization, investigation, validation, visualization, writing - original draft, writing - review & editing. **Usleber E**: Funding acquisition, project administration, resources, supervision, writing - review & editing.

Received: 14 December 2021 | Revised: 21 January 2022 | Accepted: 12 February 2022

DOI: 10.1111/1750-3841.16106

Journal of Food Science WILEY

FOOD MICROBIOLOGY AND SAFETY

Microbiological and mycotoxicological analyses of processed cereal-based complementary foods for infants and young children from the German market

Christina Rehagel | **Ömer Akineden** | **Ewald Usleber**

Dairy Sciences, Institute of Veterinary Food Science, Justus-Liebig University Giessen, Giessen, Germany

Correspondence
Christina Rehagel, Dairy Sciences, Institute of Veterinary Food Science, Justus-Liebig University Giessen, Ludwigstrasse 21, D-35390 Giessen, Germany.
Email: Christina.Rehagel@vetmed.uni-giessen.de

Abstract

This study investigated several food safety criteria in 38 different commercial products of processed cereal-based foods (PCF) from the German market. Microbiological assessment, followed by 16S RNA gene sequencing of suspect colonies, included aerobic mesophilic bacteria, moulds, *Enterobacteriaceae*, *Cronobacter* spp., and presumptive *Bacillus cereus*. Mycotoxin analyses were performed by enzyme immunoassays for deoxynivalenol (DON), zearalenone (ZEN), T-2/HT-2 toxins (T-2/HT-2; oat containing products only), ergot alkaloids (EA), and alternariol (AOH). No violative result above existing European Union regulations or international guidelines was obtained. Most samples had very low aerobic mesophilic cell counts ($<2.0 \times 10^1$ CFU/g), the maximum was 9.6×10^2 CFU/g. A few samples contained low numbers of opportunistic pathogens, most notably *Cronobacter sakazakii*, *Acinetobacter* spp., *Pantoea* spp., and enterotoxigenic *Bacillus wiedmannii*. Levels of mycotoxin contamination were very low, well below European Union maximum limits. DON was found in 10 samples, at levels of 9–35 µg/kg. T-2/HT-2 were found in all 15 oat-based products (1–8 µg/kg). All samples were negative for ZEN and EA. A high number ($n = 25$) of samples yielded weakly positive results for the nonregulated AOH (0.4–2 µg/kg), but just three samples exceeded a level of 1 µg/kg. No relationship between cereal composition and analytical findings for microbiological parameters and mycotoxins could be found. As long as PCF meals are freshly prepared and consumed immediately after preparation, the risk from sporadically occurring opportunistic bacteria appears to be minimal.

KEYWORDS
enzyme immunoassay, infant food, mycotoxins, pathogenic bacteria, processed cereal-based foods

1 | INTRODUCTION

Complementary feeding is defined as the period when complementary foods are given together with either breast milk or formula or both. Typically beginning at an age between 4 and 6 months, complementary foods are gradually introduced into the diet of infants, and commercial cereal-based foods play a major role during this period (EFSA, 2019). Processed cereal-based foods (PCF) are defined as containing simple or mixed cereals, which are ready to eat after reconstitution with milk or water (EU, 2013). PCFs are not sterile products, and microbial contamination of cereal grains may occur at various stages of production (Los et al., 2018). With a total revenue in 2020 of, for example, 6.4 billion US$ (United States) and 0.6 billion US$ (Germany), "baby food" (total) constitutes just a minor (≈0.5%) part of the total revenues of food industry in industrialized countries (Pham, 2021). No market data for PCF could be obtained from published literature. However, infant food in general is a highly sensitive market segment, which has to deal with the vulnerability of babies, and with the attitude of parents with regard to food quality and safety. Therefore, the number of companies producing and distributing such products is quite small, just a few different brands dominate the German market, and different brands may in fact have be produced in the same factory.

In contrast to commercial milk-based powdered infant formulae, pathogenic bacteria in PCFs are not specifically regulated within the European Union, except of the more general commitments arising from the "precautionary principle." For example, notorious pathogenic/toxigenic bacteria occurring in cereals are *Cronobacter* spp. and *Bacillus cereus* (Akineden et al., 2015; Kim et al., 2011; Lou et al., 2019), both regulated in infant formulae, but not in PCF, by the European Commission (EC, 2005).

Concerning mycotoxins, a substantial reduction may be achieved during cereal processing for some compounds, but not all mycotoxins can be completely removed (Karlovsky et al., 2016). Maximum levels (ML) have been set for some mycotoxins in PCFs, including aflatoxins (0.1 µg/kg), ochratoxin A (OTA, 0.5 µg/kg), deoxynivalenol (DON, 200 µg/kg), zearalenone (ZEN, 20 µg/kg), fumonisins (200 µg/kg), and, most recently, ergot alkaloids (EA, sum of 12 congeners, 20 µg/kg) (EC, 2006b). With regard to T-2/HT-2 toxins (T-2/HT-2), an "indicative level" of 15 µg/kg, from which onwards further investigations should be performed, was published for PCFs for infants and young children (EC, 2013). No specific regulations have been issued for alternariol (AOH), or other *Alternaria* toxins, in any country of the world. However, risk and exposure assessments published by the European Food Safety Authority (EFSA), underlined the need to further consider AOH as a relevant mycotoxin, and established a preliminary "threshold of toxicological concern" (TTC) of 2.5 ng/kg body weight (b.w.) and day (EFSA, 2011, 2016).

Although the importance of commercial PCF for infant nutrition certainly is high in industrialized countries, the number of published studies dealing with the microbiological and mycotoxicological quality of such products is surprisingly small, and just one study dealt with both (Assunção et al., 2021).

Surveys on PCF studying microbiological criteria with a broader scope are rare (Kim et al., 2011), but *Cronobacter* spp. (Kim et al., 2011; Ziver et al., 2020) and *B. cereus* (Assunção et al., 2021) have been detected with some frequency. The number of published mycotoxin surveys in PCF is also limited, and even fewer recent data are available (Mallmann et al., 2020). Most studies so far included DON, which was found in up to 50% of PCF samples worldwide (Assunção et al., 2018; Braun et al., 2020; Herrera et al., 2019; Juan et al., 2014; Oueslati et al., 2018; Pereira et al., 2015). The average DON contamination was at 10–100 µg/kg, but in some cases exceeded 200 µg/kg. Depending on the type of cereal, other mycotoxins have been reported with varying frequency over the last 20 years. T-2/HT-2 were predominantly found in oat-containing PCF (Al-Taher et al., 2017; Assunção et al., 2018; Braun et al., 2020; Gotthardt et al., 2019; Juan et al., 2014; Oueslati et al., 2018; Pereira et al., 2015). For AOH and EA, just a small number of published studies is available (Lombaert et al., 2003; Mulder et al., 2015; Reinhard et al., 2008; Scott et al., 2012). Gotthardt et al. (2019) analyzed 25 samples of PCF from the German market for AOH and five other *Alternaria* toxins, the most abundant compound was tenuazonic acid, while low levels (0.76–7.17 µg/kg) of AOH were detected in just six samples.

Considering the scarcity of analytical data, the aim of this study was to elucidate the safety of PCF from the German market with regard to microbiological criteria and mycotoxin contamination, including both known opportunistic pathogens and notorious mycotoxins, plus some less well-studied parameters (Gram-negative bacteria, presumptive *B. cereus*, EA, AOH).

2 | MATERIALS AND METHODS

2.1 | Sample materials and sampling

Between November 2019 and March 2020, 38 dairy-free samples of PCF, recommended age of consumption "after the fourth month" up to "after the sixth month," were purchased from local retail stores, drugstores, and specialized organic retail stores in the area of Giessen, Germany. According to product labels, the samples originated from 12 companies (trade names), most were labeled as

German produce, but 10 samples were from four other countries within the European Union. The samples were collected with the aim that all dairy-free PCF brands from all producers were included in the study. The majority of PCF products is marketed countrywide, so the collection of samples can be attributed as typical for dairy-free PCFs as available from the German market. Nowadays, all brands of PCFs available in Germany are labeled as of "organic produce." Single-grain products ($n = 25$) contained millet, spelt, oats, wheat, barley, rye, rice, or maize. Multigrain products ($n = 13$) contained at least two, up to seven, of these cereals. All products were supplemented with thiamine (vitamin B_1) according to Commission directive 2006/125/EC (EC, 2006a). All products were available in dry form, either as powder or as granulate material, ready for consumption after the addition of 10 volumes of milk, water, or milk diluted with water (1 + 1, by volume). All samples, which contained one to two portions (150–250 g each) of PCF in sealed sachets (paper or aluminum-coated foil), encased in a cardboard package, were stored dry at room temperature until testing. Before opening, each sachet was manually shaken to enhance homogeneity of the material thoroughly homogenized by swiveling the package. The test portion for microbiological analyses was taken out of this sachet under sterile conditions. For microbiological analysis, approximately 50 g were taken from each package under antiseptic conditions, and transferred to a sterile sealable glass bottle. The remaining material was filled into sealable plastic cans and used for mycotoxin analysis.

2.2 | Mycotoxin analysis

2.2.1 | Chemicals, reagents, and buffers

Ergometrine (synonym: ergonovine), ergocristine, DON, and ZEN were purchased from Sigma-Aldrich (Taufkirchen, Germany). AOH was obtained from Cayman Chemicals (Hamburg, Germany). T-2 toxin was from Biopure (Tulln, Austria). Ergotamine D-tartrate (Fluka®) and methanol (Riedel-de Haën®) were from Honeywell (Charlotte, NC, USA). Acetonitrile and ethyl acetate were purchased from Merck (Darmstadt, Germany). All reagents used were at least of analytical grade. For the analysis of EA, a toxin standard mixture of ergometrine, ergocristine, and ergotamine D-tartrate in a ratio of 1:10:14 (w/w/w; Gross et al., 2018) with a total alkaloid concentration of 25 µg/ml was prepared. All mycotoxin stock solutions (except T-2/HT-2) were checked for purity and correct concentration by UV spectroscopy (Shimadzu, Duisburg, Germany), using published spectra and absorption coefficients (Cole et al., 2003). Phosphate-buffered saline (PBS) contained 6.79 g of NaCl, 1.47 g of Na_2HPO_4, and 0.43 g of KH_2PO_4 in 1 L of a. dest. (0.01 mol/L, pH 7.3). For AOH extraction, PBS consisted of 6.79 g of NaCl, 2.94 g of Na_2HPO_4, and 0.86 g of KH_2PO_4 in 1 L of a. dest. (pH 7.2). Sodium bicarbonate buffer contained 1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ in 1 L of a. dest. (0.05 mol/L, pH 9.6). Washing solution contained 8.5 g NaCl and 0.25 ml of Tween in 1 L of a. dest. For the enzyme substrate/chromogen solution, H_2O_2-citrate buffer (8.3 g citric acid, 49 ml KOH (1 mol/L), 72 µl 30% aqueous H_2O_2, 160 ml a. dest; pH 3.9) and TMB solution (50.4 mg 3,3′,5,5′-TMB, 1 ml acetone, 9 ml methanol) were used. The enzyme substrate/chromogen solution was prepared according to Ackermann et al. (2011). Shortly before use, 0.5 ml of TMB was added to 10 ml of H_2O_2-citrate buffer.

2.2.2 | Sample extract preparation

In general, sample extract preparation for all mycotoxins was performed as described by Liesener et al. (2010), except for AOH which was extracted by a modification of the procedure described by Ackermann et al. (2011). All procedures had to be adopted to meet the requirements of the highly absorptive PCF matrix. All primary extractions were done with 5 g of sample material mixed with 50 ml of solvent in a beaker and magnetic stirring at full speed (400 rpm) for 30 min. For some toxins, extraction was followed by a centrifugation step (10 min at 3000 × *g* for DON and ZEN; 4 min at 11,000 × *g* for EA). Filtration was done using paper filters.

T-2/HT-2 were extracted with 50 ml of water containing 70% methanol. A 2 ml portion of the filtered extract was mixed with 2 ml of distilled water and extracted twice by liquid–liquid partitioning with each 3 ml portions of ethyl acetate. The two ethyl acetate phases were removed after centrifugation (3000 × *g*, 15 min) and combined, then the solvent was evaporated at 50°C in a rotary evaporator. The residue was dissolved with 0.2 ml of methanol and 1.8 ml of PBS (pH 7.3), using ultra-sonication in a water bath for 2 min. This extract was mixed with 1 ml of n-heptane on a wrist-action shaker. The phases were separated by centrifugation (3000 × *g*, 15 min). The lower aqueous phase was collected and analyzed by enzyme immunoassay (EIA) either directly (sample dilution factor: 10), or after dilution with PBS (pH 7.3) containing 10% methanol.

DON was extracted essentially by the same procedure as for T-2/HT-2, except that extraction solvent was PBS containing 10% methanol, and that no extract defatting step with n-hexane was necessary. After rotary evaporation, the residue was dissolved with 1 ml of PBS (pH 7.3). This extract was analyzed either directly (sample dilution factor: 5), or after dilution with PBS (pH 7.3).

ZEN was extracted with distilled water containing 84% acetonitrile. After centrifugation and filtration, 100 µl of the filtrate was mixed with 1.58 ml PBS (pH 7.3) to obtain a 5% acetonitrile/PBS solution for EIA analysis (sample dilution factor: 168). Further dilutions were made with 5% acetonitrile/PBS.

EAs were extracted with PBS pH 6.0 containing 60% acetonitrile and stirred for 30 min on a magnetic stirrer (400 rpm). After the solid particles had settled, 2 ml of this extract were transferred into a 2 ml Eppendorf vial and centrifuged (11,000 × *g*, 4 min, 20°C). For EIA analysis, 100 µl of this extract was mixed with 0.9 ml PBS, pH 6.0 (sample dilution factor: 100). Further dilutions were made with 5% acetonitrile/PBS pH 6.0.

AOH was extracted with PBS containing 70% methanol, the apparent pH value was adjusted to approximately 7.0 with 3 mol/L NaOH. The extract was filtrated and 2 ml was mixed with 2 ml of distilled water. The mixture was extracted twice by liquid–liquid partitioning with each 3 ml of ethyl acetate followed by centrifugation. The two organic phases were pooled, the solvent evaporated, and the residue dissolved with 1 ml of PBS for 2 min by ultrasonication in a water bath. The resulting extract was analyzed either directly (sample dilution factor: 5), or after dilution with PBS.

Recovery was tested by adding toxin standard solutions to dry sample materials before extraction. Three different toxin levels, and three different sample materials per level, were tested for each mycotoxin. The lowest concentration used for fortification of samples was 3–10 times the calculated LOD of each method. T-2/HT-2 were analyzed only in products, which were labeled as containing oats ($n = 15$). Further, the test samples, which gave the highest toxin results in the enzyme immunoassays (EIAs) for DON, T-2/HT-2, and AOH, were each spiked with the respective toxin at the same level, to double the natural amount of toxin, and then were reanalyzed.

2.2.3 | EIA analyses

All competitive EIAs were performed as microtiter plate (MaxiSorp, Nunc, Roskilde, Denmark) assays as described previously for DON, ZEN, T-2/HT-2, EA, and AOH (Gross et al., 2018). Some important EIA test characteristics are compiled in Table 1. Four replicate wells were tested for each standard concentration and for each dilution of sample extract solution. The resulting EIA absorbance values at 450 nm were measured with a model Sunrise microplate reader (Tecan, Crailsheim, Germany) and evaluated by Magellan EIA calculation software (Tecan). All values were then standardized as percent relative absorbance of the blank (B_0), by dividing the mean absorbance values of standard or sample solutions through the absorbance of B_0, multiplying by 100 ($B/B_0 \times 100$). The standard curve detection limits were set as cut-off values of 70% –80% as described for each EIA in the original studies. Recovery was routinely checked, before the start and during the analyses of each series, by addition of 50–100 µl of toxin standard solution at appropriate concentrations to dry samples before extraction, and allowing for the solvent to evaporate. Each three samples with different matrix composition were tested for each test system. The calculated mean detection limit of each test system for PCF was derived from the mean standard curve detection limit, multiplied by the minimum sample dilution factor, without considering recovery. All toxin concentrations for naturally contaminated samples were reported without correction for the analytical recovery.

2.3 | Microbiological analyses

Microbiological parameters included nonspecific hygiene indicators (aerobic mesophilic plate count (APC), *Enterobacteriaceae*, moulds) and specific pathogenic (*Cronobacter* spp.) or enterotoxin-producing enterotoxigenic bacteria (presumptive *B. cereus*).

All sample materials were prepared for analysis according to ISO 6887-4:2017. For all tests, a 10 g test portion was mixed with 190 ml of 0.1% sterile peptone water (Oxoid, Wernigerode, Germany) in a sterile plastic bag and homogenized in a paddle blender (Stomacher). Two further decimal dilutions (2×10^{-2}; 2×10^{-3}) were prepared with sterile peptone water (Oxoid). Each 100 µl of the original homogenate and the two dilutions were spread onto two plates and 1 ml of the original homogenate onto four plates of the appropriate media. All sample homogenates were analyzed in duplicate. Taking into account the minimum dilution factor, the detection limit was 2.0×10^1 colony forming units (CFU)/g. All incubation of plates were done under aerobic conditions.

The number of aerobic mesophilic bacteria was determined on plate count agar (PC agar, Oxoid) plates according to ISO 4833-2:2014, incubation was at 30°C for 24–48 h.

The enumeration of *Enterobacteriaceae* was done on Violet Red Bile Glucose (VRBG) agar plates according to ISO 21528-2:2017, incubation was 37°C for 24–48 h.

Mould counts were determined on Sabouraud agar plates with chloramphenicol 0.5 g/L (Oxoid), with an incubation at 25°C for 3–5 days.

Presumptive *B. cereus* were isolated by surface plating on polymyxin egg yolk mannitol bromthymol blue agar, PEMBA (Oxoid) according to ISO 7932:2004, and incubation at 30°C for 24 h. Colonies showing typical

TABLE 1 Overview of enzyme immunoassays (EIAs) used in this study and test performance in relationship to benchmark values

EIA system			Test sensitivity achieved		
Name	Known relevant cross-reactions	Reference	Mean LOD, standard curve cut-off value (ng/ml)	LOD in PCF (µg/kg)	Benchmark value, µg/kg
DON	DON and its 8-ketotrichothecene analogues	(Usleber et al., 1991; Curtui et al., 2003)	2 ± 0.5	10	200[a]
ZEN	ZEN and their analogues	(Usleber et al., 1992; Seidler, 2007)	0.04 ± 0.02	7	20[a]
T-2/HT-2	T-2, HT-2	(Esgin et al., 1989)	0.05 ± 0.01	0.5	15[b]
EA	All EAs and isomers	(Gross et al., 2018)	0.3 ± 0.05	30	20[a]
AOH	None	(Ackermann et al., 2011)	0.4 ± 0.05	0.3	1[c]

[a]EC (2006b).
[b]EC (2013).
[c]Estimated from threshold of toxicological concern (TTC) value (2.5 ng/kg body weight (b.w.) and day; EFSA, 2011), assuming a b.w. of 10 kg and a daily consumption of 25 g dry product.
Abbreviations: DON, deoxynivalenol; ZEN, zearalenone, EA, ergot alkaloids; AOH, alternariol.

morphology, blue color, and a precipitation zone were transferred to Columbia agar supplemented with 5% of sheep blood (30°C, 24 h). Isolates identified as presumptive *B. cereus* on PEMBA were tested for toxin production (hemolysin BL [Hbl]; nonhemolytic enterotoxin [Nhe]) with the Duopath® Cereus Enterotoxins immunoassay (Merck).

Cronobacter spp. were qualitatively detected by method ISO 22964:2017. A 10 g portion of the sample was reconstituted with 190 ml buffered peptone water (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) according to ISO 6887-4:2017, and pre-enriched at 37°C for 18 h. Then, 0.1 ml was added to 10 ml of *Cronobacter* Selective Broth (CSB; Oxoid Thermo Fisher) and cultured at 41.5°C for 24 h. A 10 µl portion was streaked onto Chromogenic *Cronobacter* Isolation agar (CCI Agar; Oxoid Thermo Fisher) with a loop, and incubated at 41.5°C for 24 h. Colonies which were identified as tentative *Cronobacter* were further characterized by a commercial biochemical identification system (API32E; bio-Mérieux, Marcy l'Etoile, France), and then identified on species level by analyzing the *fusA* gene sequences as described previously by Akineden et al. (2017).

Further characterization of randomly chosen isolates was done if a noticeable colony growth was observed on PC agar, VRBG agar, PEMBA, or CCI Agar, excluding typical aerobic spore forming bacteria on PC agar, which frequently grew on PC agar in low densities. One or more typical, morphologically distinct colony was selected and purified by streaking onto Columbia blood agar. The genomic DNA was extracted from single colonies with a commercial test system (DNeasy blood and tissue kit, Qiagen). Species identification of isolates was done by amplification and sequencing of the 16S rRNA gene (Kuhnert et al., 1996). For species identification, sequences were compared using the "Basic Local Alignment Search Tool" (BLAST) at the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3 | RESULTS AND DISCUSSION

3.1 | Microbiological analyses

The vast majority of samples were negative for *Enterobacteriaceae*, moulds, and for presumptive *B. cereus* (Table 2). Each sample yielded visible colony growth on VRBG (sample #6) and PEMBA (#20), respectively. Four samples had mould counts at the detection limit of ($2.0–4.0 \times 10^1$ CFU/g), and only one sample (#17) was moderately positive for moulds at 2.0×10^2 CFU/g. On CCI agar, two samples (#6, #17) resulted in colony growth, indicative for *Cronobacter* spp.

With regard to aerobic mesophilic bacteria, the majority of samples (24 of 38) were negative, six were weakly positives ($10^1–10^2$ CFU/g). However, eight samples had colony counts of $\geq 10^2$ CFU/g, highest result was obtained for sample #6 (9.6×10^2 CFU/g). As far as we know, no specific regulation for aerobic colony counts in PCF exist, neither in the European Union nor elsewhere. However, this parameter has been addressed as a "useful testing" criterion by the International Commission on Microbiological Specifications for Foods (ICMSF), and limits/g in a range of $m = 1 \times 10^3 – 5 \times 10^3$ to $M = 1 \times 10^4 – 5 \times 10^4$ have been suggested (ICMSF, 2011). The ML obtained in our study were 1–2 orders of magnitude lower than that,

TABLE 2 Quantitative results for aerobic mesophilic bacteria, Enterobacteriaceae, moulds, and presumptive *Bacillus cereus* in processed cereal-based foods (PCF) ($n = 38$), number of sample per interval

CFU/g interval	Aerobic mesophilic bacteria	*Enterobacteriaceae*	Moulds	Presumptive *Bacillus cereus*
Negative, $< 2.0 \times 10^1$	24	37	33	37
10^1–$< 10^2$	6	0	4	1[b]
10^2–$< 10^3$	8	1[a]	1	0

[a]Identified as *Acinetobacter baumannii* (non-Enterobacteriaceae).
[b]Identified as *Bacillus wiedmannii*.

TABLE 3 Identification of isolates from plate count agar (PC agar) of processed cereal-based foods (PCF) based on partial sequencing of the 16S rRNA gene

No. of sample and isolate	No. of isolate analyzed	Phylogenetic affiliation	Designation of isolate	Sequence length (bp)	Identity % (query coverage)	Closest species (NCBI accession number)
3	3	*Moraxella osloensis*	BBK 10/20	1304	99.23 (99)	*Moraxella osloensis* DSM 6998^T (NR_104936.1)
		Moraxella osloensis	BBK 14/20	1304	99.69 (100)	*Moraxella osloensis* DSM 6998^T (NR_104936.1)
		Moraxella osloensis	BBK19/20-pca2	1310	99.54 (99)	*Moraxella osloensis* DSM 6998^T (NR_104936.1)
2	2	*Acinetobacter baumannii*	BBK 6/20	1308	99.85 (99)	*Acinetobacter baumannii* DSM 30007^T (NR_117677)
		Acinetobacter nosocomialis	BBK12/20-cci	1312	99.62 (99)	*Acinetobacter nosocomialis* DSM 102856^T (NR_117931.1)
1	1	*Pantoea brenneri*	BBK17/20	1181	99.41 (100)	*Pantoea brenneri* DSM 24232^T (NR_116748.1)
1	1	*Microbacterium zeae*	BBK13/20	1294	99.07 (99)	*Microbacterium zeae* DSM 100750^T (NR_149816.1)
1	1	*Chryseobacterium hominis*	BBK18/20-pca2	1238	98.54 (100)	*Chryseobacterium hominis* DSM 30866^T (NR_042517.2)
1	1	*Janibacter melonis*	BBK19/20-pca1	1293	99.61 (100)	*Janibacter melonis* DSM 16063^T (NR_025805.1)
1	1	*Micrococcus aloeverae*	BBK18/20-pca1	1295	99.77 (99)	*Micrococcus aloeverae* DSM 27472^T (NR_134088.1)
4	1	*Bacillus subtilis*	BBK12/20-pca	1324	99.85 (99)	*Bacillus subtilis* DSM 10^T (NR_027552.1)

supporting the conclusion that for this parameter, a good overall quality of all products could be assumed. Nevertheless, each one to two characteristic isolates representing the dominant type of colony morphology per PC agar plate, from a total of 14 positive samples, were further identified, using biochemical methods and 16S rDNA sequencing, to obtain some preliminary information about the spectrum of bacterial species in PCF (Table 3). The dominant isolates from four samples were identified as *Bacillus subtilis*. For colony growth on the remaining 10 PC agar plates, non-spore forming bacteria dominated. The identified species presented a very diverse spectrum of bacteria and included Gram-negative (*Acinetobacter nosocomialis*, *Chryseobacterium hominis*, *Moraxella osloensis*, *Pantoea brenneri*) and Gram-positive (*Janibacter melonis*, *Microbacterium zeae*, *Micrococcus luteus* [syn. *M. aloeverae*]) species (Table 3). All these species have been isolated from various environmental habitats including plants, but also from infant formulae and from clinical specimen, some as commensal bacteria, but some have been involved in nosocomial human infectious diseases (Rajilić-Stojanović & de Vos, 2014).

Species identification after 16S RNA gene analysis of one suspect colony growth on VRBG (sample #6) revealed that this was not caused by a member of the *Enterobacteriaceae* family but by *Acinetobacter baumannii*.

Interestingly, the same species was identified from plate count agar of sample #6, indicating that *A. baumannii* was a major bacterial species in this particular sample material. Furthermore, species identification of suspect *Cronobacter* spp. from CCI of sample #6 showed that it was *C. sakazakii*, thereby confirming co-contamination with both opportunistic pathogens, which both have a history of causing foodborne infectious disease in newborns and infants <6 months of age (Amorim & Nascimento, 2017; Taylor et al., 2021).

Cronobacter spp. was isolated from the CCI plate of a second sample (#17), and this isolate also turned out to be *C. sakazakii*. Another isolate from this sample, collected from PC agar, was identified as *Pantoea brenneri*. Most *Pantoea* spp. are ubiquitous on plants and in water, but also have been frequently isolated from infant formulae (Estuningsih et al., 2006), and have been associated with very rare but severe cases of septicaemia in newborn or immunocompromised infants (Bergman et al., 2007).

Cronobacter spp., specifically *C. sakazakii*, probably have been the most notorious foodborne pathogens in commercial, milk-based powdered infant formulae in the last decades (Muytjens et al., 1988; Taylor et al., 2021). *Cronobacter* spp. have also been isolated from various cereal-based foods (Akineden et al., 2017; Friedemann, 2007; Lou et al., 2019; Silva et al., 2019), but little information about the frequency in PCF is available. In our study, colony forming units of *Cronobacter* spp. were obtained only by using 10 g test portions, and including a pre-enrichment step. Low levels of contamination, usually lower than 1 CFU/g (Al-Holy et al., 2011; Muytjens et al., 1988), seem to by typical for *Cronobacter* in dried foods. In the European Union, PCF for infants and young children are regulated under the Commission Directive 2006/125/EC (EC, 2006a), but this does not include microbiological criteria. *Cronobacter* spp., presumptive *B. cereus*, and *Enterobacteriaceae* in milk-based commercial formulae for infants <6 months of age are covered by European Union regulation No. 2073/2005 (EC, 2005), but this regulation does not apply to PCF.

Four samples yielded a weakly positive result for moulds, all near the detection limit (2.0×10^1 CFU/g) of the method. By microscopy, all were tentatively identified as *Penicillium* spp. No further attempt was made to identify and characterize these colonies at the species level.

Only one sample (#20) yielded colony growth on PEMBA (2.0×10^1 CFU/g), indicative for presumptive *B. cereus*. Further 16S rDNA sequence analysis identified this isolate as *Bacillus wiedmannii*, which is a member of the *B. cereus* group (Miller et al., 2016). This isolate also showed hemolytic activity on blood agar and was positive for Nhe and Hbl in the Duopath® Cereus Enterotoxins test (Merck). Toxigenic strains of the *B. cereus* group species have previously been reported in infant foods, including cereal-based products (Kim et al., 2011; Sadek et al., 2018). Severe outbreaks of intoxication associated with enterotoxigenic *B. cereus* spp. in food consumed by children have been reported (Delbrassinne et al., 2015; Dierick et al., 2005), but these cases were also characterized by improper food handling. At such a low level of contamination, which would have been well below even the European Union requirements for infant formulae (EC, 2005), this does not appear to be a food safety issue.

Summarizing the results of the microbiological analyses, all samples had low or very low colony counts, and five out of 38 PCF products were found to be positive, at low level, for one or two bacterial species that have been associated with rare cases of newborn or preterm infant disease. Nine other samples contained moulds or commensal bacteria at low numbers. We agree with the opinion expressed by the ICMSF (2011) that findings in such a range cannot be regarded as a direct threat to the health of infants ≥4 months of age, as long as the product is prepared and handled according to the recommendations. Further, no clear association between a specific PCF ingredient or mixture of ingredients and the occurrence of specific bacteria could be detected, also because the majority of products contained mixed cereals. These products yielded most remarkable findings, including both *C. sakazakii* isolates, *A. baumannii*, *P. brenneri*, and *B. wiedmannii* (Table 4).

3.2 | Mycotoxin analyses

3.2.1 | Method validation

All PCF products under study were dried powdery or granular products of similar appearance, the ingredients presented a wide variety of different cereals as ingredients or ingredient mixtures. The majority of products in this study contained cereal mixtures, similar as observed from the display of products in eight local retail shops. Product labels listed up to seven different cereals, providing multiple potential sources for mycotoxin contamination. Out of the *Fusarium* mycotoxins under study, DON and ZEN were analyzed in all samples, T-2/HT-2 were analyzed in oat-containing products only, because oats appear to be by far the most relevant source in middle European cereals (EFSA, 2017). For each mycotoxin EIA, the mean limit of detection (LOD) as indicated by Liesener et al. (2010) was checked and verified, using spiked PCF sample material. The recovery experiments showed that the mean recovery rates for DON, T-2/HT-2, and AOH in spiked samples were in the range of 69%–120% (Table 5). For EA and ZEN, which were also analyzed in all samples, higher recoveries ranging from 115% to 160% were

TABLE 4 Compilation of samples which yielded highest or else remarkable results, with regard to either microbiological contamination or maximum mycotoxin levels

	Sample #						
Parameter	**6**	**12**	**17**	**20**	**24**	**30**	**34**
Composition	Oats, wheat, barley, spelt, rye	Wheat	Oats, einkorn wheat, barley, rye	Oats, wheat, rye, barley, spelt	Rice, maize, sorghum	Wheat, oats, rye, barley, sorghum, rice, maize	Wheat
Recommended age, months	≥6	≥4	≥6	≥6	≥4	≥6	≥4
Microbiology							
Aerobic mesophilic bacteria, CFU/g	9.6×10^2	3.6×10^2	1.2×10^2	$<2.0 \times 10^1$	$<2.0 \times 10^1$	$<2.0 \times 10^1$	$<2.0 \times 10^1$
Specific isolates	*C. sakazakii, A. baumannii*	*A. nosocomialis, B. subtilis*	*C. sakazakii, P. brenneri*	*B. wiedmannii*	–	–	–
Mycotoxin EIA results, μg/kg							
DON	<9	<9	15	<9	<9	11	35
T-2/HT-2	4	n.a.	5	5	n.a.	8	n.a.
AOH	1	<0.3	0.5	0.7	2	2	<0.3

Abbreviations: AOH, alternariol; DON, deoxynivalenol; EIA, enzyme immunoassay; n.a., not analyzed.

TABLE 5 Recovery of mycotoxins from artificially contaminated processed cereal-based foods (PCF) samples. Each level was spiked in triplicate

		Toxin found	
Test system for	**Spiked level (μg/kg)**	**Mean recovery (%)**	**RSD (%)**
DON	30	69	4
	50	78	4
	150	70	9
ZEN	20	155	9
	50	133	10
	100	127	5
T-2/HT-2	10	99	10
	20	120	19
	50	96	17
EA	100	160	29
	200	130	18
	500	115	9
AOH	3	72	18
	5	85	3
	10	75	10

Abbreviations: AOH, alternariol; DON, deoxynivalenol; EA, ergot alkaloids; RSD, relative standard deviation; ZEN, zearalenone.

determined. The high recoveries obtained for ZEN may be explained by some remaining matrix interference in the three selected materials, because the blank materials used for spiking were clearly below the LOD but yielded absorbance values of 93%–98% $B/B_0 \times 100$. Likewise, the high recovery rates for EA may also be explained by some remaining matrix effects. Since both tests yielded toxin-negative results for all samples, no attempt was made to further improve sample extract preparation. Further studies will aim at optimizing the overall analytical strategy, including sample extraction and confirmation of positive results by, for example, an LC-MS/MS reference method.

Relative standard deviations (RSDs) of ≤10% were found for DON and ZEN, whereas RSDs <20% could be achieved for all other mycotoxins except for the 100 μg/kg spiking level of EA (29%). Further, three samples containing each of the highest toxin level (Table 4) of DON (#34), T-2/HT-2 (#30), and AOH (#24) were spiked with the respective toxin and reanalyzed. After subtracting the measured toxin content of the nonspiked sample, recoveries of 77%–106% were obtained, which demonstrates a toxin-dependent additive effect and further indicates the suitability of the EIA methods.

While the sample extraction methods, and the overall method performance were considered to be sufficient, the relative cross-reactivities of the EIAs, specifically for T-2/HT-2 and EA may present some underestimate of the true toxin content, while the DON and AOH results could be an overestimate due to reactivity with toxin analogues. The LOD of the EIA method for EAs (30 μg/kg) did not fully meet the recently published European Union ML for this group of toxins in PCF (20 μg/kg), but still are at a very similar level.

3.2.2 | Mycotoxin frequency and levels

All samples, even rye-containing products, yielded clearly EA-negative results, which indicates that this group of mycotoxins does not play a relevant role in PCF from the German market (Table 6). EAs in cereals, even if present in the harvested lots, can easily be removed during the cleaning process, allowing selection of low-contamination batches for production of PCF. It could also be assumed that PCF producers were already aware of "soon to come" European Union regulations for EAs in 2019/2020. This could explain why the contamination situation as found in our study was better than that reported in a few previous studies. For example, breakfast cereals, biscuits, and cookies from the Dutch market 2010–2014 had mean total EAs levels of 10 μg/kg, with some products exceeding 100 μg/kg (Mulder et al., 2015). Apparently, dedicated PCF products were not included in this study. Similar results were reported in a 1997–1999 survey for Canadian products (Lombaert et al., 2003).

Like EAs, ZEN was also not detected in any sample. Although ZEN is a common contaminant in most cereals at the time of harvest, it is largely eliminated during the grain cleaning processes. Our results on the absence of ZEN at <7 μg/kg are supported by others who also found no ZEN, or just traces near 1 μg/kg, in PCF from other European countries (Braun et al., 2020; Juan et al., 2014), while slightly higher concentrations were reported for such products from the United States (Al-Taher et al., 2017).

DON was found with relatively high frequency (26%) but at low levels (maximum concentration: 35 μg/kg) in PCF products, regardless of cereal composition (Table 6). While most samples were negative (<9 μg/kg), the majority of positive samples contained levels of just DON, probably the most frequent *Fusarium* toxin in cereals in Germany, and, in contrast to ZEN, removal during cereal processing is not fully efficient (Karlovsky et al., 2016), which may explain our findings. Similar findings have been reported by others (Herrera et al., 2019; Juan et al., 2014; Pereira et al., 2015).

T-2/HT-2 were found in all 15 oat-containing products, but again at very low concentrations between 1 and 8 μg/kg (Table 6). This was not unexpected, as previous surveys have shown that oat is the major, if not only, relevant cereal in Europe in aspects of T-2/HT-2 contamination (Curtui et al., 2009; Gottschalk et al., 2009; Kirinčič et al., 2015). None of the samples exceeded the guideline value of 15 μg/kg. Our results are consistent with the findings of Al-Taher et al. (2017), who reported low levels of T-2/HT-2 (<10 μg/kg) in oat-based and mixed-grain infant cereals from the U.S. market.

It was not surprising that a considerable part of the samples (66%) from each of the six product categories was positive for AOH (0.4–2 μg/kg) (Table 6), because AOH is the most frequent occurring *Alternaria* mycotoxin in food (EFSA, 2011). Similar levels of AOH (<10 μg/kg) in single grain as well as in multi-grain baby foods had also been reported by Scott et al. (2012) and Gotthardt et al. (2019). However, the AOH levels in positive samples consistently were very low. In the absence of a full toxicological risk assessment, it is difficult to evaluate the relevance of these findings. Applying (i) the TTC recommended for AOH by EFSA (2.5 ng/kg b.w. and day; EFSA, 2011), (ii) assuming that 25 g PCF (dry product) is a reasonable daily serving size, and (iii) using a b.w. range (6–12 months) of 5–10 kg, the critical AOH concentration in PCF would be at 0.5–1 μg/kg. This means that several samples would have approached or even exceeded this critical concentration, similar as estimated by EFSA in 2011 (EFSA, 2011). It has to be emphasized that the TTC approach for AOH is affected by several uncertainties, including the lack of in vivo toxicological data.

Co-occurrence of DON and AOH was observed in five samples (13%). Out of 15 oat-containing samples, which were all positive for T-2/HT-2, one additionally contained DON (oat-based product) and eight contained AOH (four oat-based products + four mixed-grain products). All three toxins were found in three oat-containing samples (mixed-grain products). This is not surprising, as the majority ($n = 7$) of these 12 co-contaminated oat-containing samples were composed of mixed cereals, providing different sources for contamination of fungal toxins, and similar

TABLE 6 Mycotoxin contamination in processed cereal-based foods (PCF) for infants and young children ($n = 38$) from the German market based on six product categories

	DON	ZEN	T-2/HT-2	EA	AOH
Spelt-based cereals ($n = 5$)					
Positive (%)	40	–	n.a.	–	60
Mean[a] ± SD	12 ± 3	<7	n.a.	<30	0.7 ± 0.2
Range[a]	11–14	<7	n.a.	<30	0.5–0.9
Wheat-based cereals ($n = 5$)					
Positive (%)	20	–	n.a.	–	40
Mean[a] ± SD	35	<7	n.a.	<30	0.7 ± 0.2
Range[a]	–	<7	n.a.	<30	0.6–0.8
Oat-based cereals ($n = 6$)					
Positive (%)	17	–	100	–	67
Mean[a] ± SD	17	<7	4 ± 1	<30	0.6 ± 0.1
Range[a]	–	<7	3–6	<30	0.4–0.7
Millet-based cereals ($n = 3$)					
Positive (%)	–	–	n.a.	–	100
Mean[a] ± SD	<9	<7	n.a.	<30	0.6 ± 0.1
Range[a]	<9	<7	n.a.	<30	0.5–0.7
Rice-based cereals ($n = 6$)					
Positive (%)	17	–	n.a.	–	33
Mean[a] ± SD	11	<7	n.a.	<30	1 ± 0.2
Range[a]	–	<7	n.a.	<30	1.1–1.5
Mixed-grain cereals ($n = 13$)					
Positive (%)	38	–	100[b]	–	85
Mean[a] ± SD	13 ± 2	<7	4 ± 2	<30	0.9 ± 0.6
Range[a]	11–15	<7	1–8	<30	0.4–2

Note: Concentration values as mean ± standard deviation (SD) expressed in μg/kg.
Abbreviations: AOH, alternariol; DON, deoxynivalenol; EA, ergot alkaloids; n.a., not analyzed; ZEN, zearalenone.
[a]Positive samples only.
[b]$n = 9$.

findings have been reported for PCF products previously (Juan et al., 2014; Zhang et al., 2018).

Concluding the results of the mycotoxin analyses, contamination frequency of PCF with DON, T-2/HT-2, and AOH was within a span, which was expected from previous studies, but levels of contamination were all in the low range, well below the ML. The complete absence of detectable levels of ZEN and EAs may be explainable by careful cereal cleaning and selection and procedures.

4 | CONCLUSION

This is the first analysis of the PCFs from the German market, and the second one worldwide (Assunção et al., 2021), in which two major food safety categories have been studied, namely microbiological quality and contamination with mycotoxins. Of course pathogenic, opportunistic, or spoilage bacteria have no direct relationship with fungal toxins. However, it may be argued that an overall deficit in maintaining effective measures to ensure food safety and quality in PCF production facilities may negatively impact both areas. Our study, although limited in sample number, included products of all major companies offering PCF products on the German market. All products fully complied with present European Union regulations of microbiological criteria and mycotoxin contamination. Very few samples (Table 4) yielded results which, in one or more parameters, surfaced the generally unremarkable data set of analytical findings. The risk from low levels of *Cronobacter* spp. and enterotoxigenic *Bacillus* spp., which are a major concern in infant formulae for preterm infants and neonates, can easily be avoided if PCF are prepared freshly for each meal and consumed immediately thereafter.

AUTHOR CONTRIBUTIONS

Christina Rehagel: Conceptualization; formal analysis; investigation; validation; visualization; writing – original draft; writing – review & editing. **Ömer Akineden**: Conceptualization; investigation; validation; visualization; writing – original draft; writing – review & editing. **Ewald Usleber**: Funding acquisition; project administration; resources; supervision; writing – review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Christina Rehagel https://orcid.org/0000-0002-1816-4391
Ömer Akineden https://orcid.org/0000-0002-0066-7054

REFERENCES

Ackermann, Y., Curtui, V., Dietrich, R., Gross, M., Latif, H., Märtlbauer, E., & Usleber, E. (2011). Widespread occurrence of low levels of alternariol in apple and tomato products, as determined by comparative immunochemical assessment using monoclonal and polyclonal antibodies. *Journal of Agricultural and Food Chemistry*, *59*, 6360–6368. https://doi.org/10.1021/jf201516f

Akineden, Ö., Murata, K. J., Gross, M., & Usleber, E. (2015). Microbiological quality of raw dried pasta from the German market, with special emphasis on *Cronobacter* species. *Journal of Food Science*, *80*, M2860–M2867. https://doi.org/10.1111/1750-3841.13117

Akineden, Ö., Heinrich, V., Gross, M., & Usleber, E. (2017). Reassessment of *Cronobacter* spp. originally isolated as *Enterobacter sakazakii* from infant food. *Food Microbiology*, *65*, 44–50. https://doi.org/10.1016/j.fm.2017.01.021

Al-Holy, M. A., Shin, J.-H., Osaili, T. M., & Rasco, B. A. (2011). Evaluation of a new enrichment broth for detection of *Cronobacter* spp. in powdered infant formula. *Journal of Food Protection*, *74*(3), 387–393. https://doi.org/10.4315/0362-028X.JFP-10-239

Al-Taher, F., Cappozzo, J., Zweigenbaum, J., Lee, H. J., Jackson, L., & Ryu, D. (2017). Detection and quantitation of mycotoxins in infant cereals in the U.S. market by LC-MS/MS using a stable isotope dilution assay. *Food Control*, *72*, 27–35. https://doi.org/10.1016/j.foodcont.2016.07.027

Amorim, A. M. B., & Nascimento, J. S. (2017). *Acinetobacter*: an underrated foodborne pathogen? *The Journal of Infection in Developing Countries*, *11*(2), 111–114. https://doi.org/10.3855/jidc.8418

Assunção, R., Martins, C., Vasco, E., Jager, A., Oliveira, C., Cunha, S. C., Fernandes, J. O., Nunes, B., Loureiro, S., & Alvito, P. (2018). Portuguese children dietary exposure to multiple mycotoxins—An overview of risk assessment under MYCOMIX project. *Food and Chemical Toxicology*, *118*, 399–408. https://doi.org/10.1016/j.fct.2018.05.040

Assunção, R., Boué, G., Alvito, P., Brazão, R., Carmona, P., Carvalho, C., Correia, D., Fernandes, P., Lopes, C., Martins, C., Membré, J.-M., Monteiro, S., Nabais, P., Thomsen, S. T., Torres, D., Pires, S. M., & Jakobsen, L. S. (2021). Risk-benefit assessment of cereal-based foods consumed by Portuguese children aged 6 to 36 months—A case study under the RiskBenefit4EU project. *Nutrients*, *13*(9), 3127. https://doi.org/10.3390/nu13093127

Bergman, K. A., Arends, J. P., & Schölvinck, E. H. (2007). *Pantoea agglomerans* septicemia in three newborn infants. *The Pediatric Infectious Disease Journal*, *26*(5), 453–454. https://doi.org/10.1097/01.inf.0000261200.83869.92

Braun, D., Eiser, M., Puntscher, H., Marko, D., & Warth, B. (2020). Natural contaminants in infant food: The case of regulated and emerging mycotoxins. *Food Control*, *123*, 107676. https://doi.org/10.1016/j.foodcont.2020.107676

Cole, R. J., Jarvis, B. B., & Schweikert, M. A. (2003). *Handbook of secondary fungal metabolites* (Vol. 3). Academic Press.

Curtui, V., Seidler, C., Dietrich, R., Märtlbauer, E., Schneider, E., & Usleber, E. (2003). Bestimmung von Deoxynivalenol in Brot und Bier. *Mycotoxin Research*, *19*, 144–148.

Curtui, V., Usleber, E., Trebstein, A., Lauber, U., Hocke, K., Dietrich, R., Märtlbauer, E., Majerus, P., Zimmer, M., Klaffke, H., Gareis, M., Betsche, T., & Langenkämper, G. (2009). *Improvement and validation of analytical methods for type A trichothecenes (T-2 toxin and HT-2 toxin) and occurrence of these mycotoxins in foods from the German market*. Bundesanstalt für Landwirtschaft und Ernährung (BLE). https://service.ble.de/ptdb/index2.php?detail_id=88841&site_key=145&stichw=trichothecene&zeilenzahl_zaehler=4#newContent

Delbrassinne, L., Botteldoorn, N., Andjelkovic, M., Dierick, K., & Denayer, S. (2015). An emetic *Bacillus cereus* outbreak in a kindergarten: detection and quantification of critical levels of cereulide toxin. *Foodborne Pathogens and Disease*, *12*(1), 84–87. https://doi.org/10.1089/fpd.2014.1788

Dierick, K., Van Collie, E., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M., & Mahillon, J. (2005). Fatal family outbreak of *Bacillus cereus*-associated food poisoning. *Journal of Clinical Microbiology*, *43*, 4277–4279. https://doi.org/10.1128/JCM.43.8.4277-4279.2005

EC (European Commission). (2005). Commission regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, *L 338*, 1–26. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R2073&from=EN

EC. (2006a). Commission directive 2006/125/EC of 5 December 2006 on processed cereal-based foods and baby foods for infants and young children (Codified version). *Official Journal of the European Union*, *L 339*, 16–35. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006L0125&from=EN.

EC. (2006b). Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, *L 364*, 5–24. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF

EC. (2013). Commission recommendation 2013/165/EU of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. *Official Journal of the European Union*, *L 91*, 12–15. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013H0165&from=EN

EFSA (European Food Safety Authority). (2011). Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA Journal*, *9*(10), 2407. https://doi.org/10.2903/j.efsa.2011.2407

EFSA. (2016). Dietary exposure assessment to *Alternaria* toxins in the European population. *EFSA Journal*, *14*(12), 4654. https://doi.org/10.2903/j.efsa.2016.4654

EFSA. (2017). Human and animal dietary exposure to T-2 and HT-2 toxin. *EFSA Journal*, *15*(8), 4972. https://doi.org/10.2903/j.efsa.2017.4972

EFSA. (2019). Appropriate age range for introduction of complementary feeding into an infant's diet. *EFSA Journal*, *17*(9), 5780. https://doi.org/10.2903/j.efsa.2019.5780

Esgin, S., Märtlbauer, E., & Terplan, G. (1989). Entwicklung und Anwendung eines enzymimmunologischen Verfahrens zum Nachweis von T-2 Toxin in Milch. *Archiv Für Lebensmittelhygiene*, *40*, 109–112.

Estuningsih, S., Kress, C., Hassan, A. A., Akineden, Ö., Schneider, E., & Usleber, E. (2006). *Enterobacteriaceae* in dehydrated powdered infant formula manufactured in Indonesia and Malaysia. *Journal of Food Protection*, *69*(12), 3013–3017. https://doi.org/10.4315/0362-028X-69.12.3013

EU (European Union). (2013). Regulation (EU) No 609/2013 of the European parliament and of the council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing council directive 92/52/EEC, commission directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, directive 2009/39/EC of the European parliament and of the council and commission regulations (EC) No 41/2009 and (EC) No 953/2009. *Official Journal of the European Union*, *L 181*, 35–56. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R0609&from=EN

Friedemann, M. (2007). *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). *International Journal of Food Microbiology*, *116*(1), 1–10. https://doi.org/10.1016/j.ijfoodmicro.2006.12.018

Gotthardt, M., Asam, S., Gunkel, K., Moghaddam, A. F., Baumann, E., Kietz, R., & Rychlik, M. (2019). Quantitation of six *Alternaria* toxins in infant foods applying stable isotope labeled standards. *Frontiers in Microbiology*, *10*, 109. https://doi.org/10.3389/fmicb.2019.00109

Gottschalk, C., Barthel, J., Engelhardt, G., Bauer, J., & Meyer, K. (2009). Simultaneous determination of type A, B and D trichothecenes and their occurrence in cereals and cereal products. *Food Additives & Contaminants*, *26*, 1273–1289. https://doi.org/10.1080/02652030903013260

Gross, M., Curtui, V., & Usleber, E. (2018). Detection of total ergot alkaloids in cereal flour and in bread by a generic enzyme immunoassay method. *Journal of AOAC International*, *101*, 618–626. https://doi.org/10.5740/jaoacint.17-0346.

Herrera, M., Bervis, N., Carramiñana, J. J., Juan, T., Herrera, A., Ariño, A., & Lorán, S. (2019). Occurrence and exposure assessment of aflatoxins and deoxynivalenol in cereal-based baby foods for infants. *Toxins*, *11*, 150. https://doi.org/10.3390/toxins11030150

ICMSF (International Commission on Microbiological Specifications for Foods). (2011). *Microorganisms in foods 8: Use of data for assessing process control and product acceptance* (1st ed). Springer. https://doi.org/10.1007/978-1-4419-9374-8_15

Juan, C., Raiol, A., Mañes, J., & Ritieni, A. (2014). Presence of mycotoxin in commercial infant formulas and baby foods from Italian market. *Food Control*, *39*, 227–236. http://doi.org/10.1016/j.foodcont.2013.10.036

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I. P., Speijers, G., Chiodini, A., Recker, T., & Dussort, P. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin Research*, *32*, 179–205. https://doi.org/10.1007/s12550-016-0257-7

Kim, S. A., Oh, S. W., Lee, Y. M., Imm, J. Y., Hwang, I. G., Kang, D. H., & Rhee, M. S. (2011). Microbial contamination of food products consumed by infants and babies in Korea. *Letters in Applied Microbiology*, *53*, 532–538. https://doi.org/10.1111/j.1472-765X.2011.03142.x

Kirinčič, S., Škrjanc, B., Kos, N., Kozolc, B., Pirnat, N., & Tavčar-Kalcher, G. (2015). Mycotoxins in cereals and cereal products in Slovenia-Official control of foods in the years 2008–2012. *Food Control*, *50*, 157–165. https://doi.org/10.1016/j.foodcont.2014.08.034

Kuhnert, P., Capaul, S. E., Nicolet, J., & Frey, J. (1996). Phylogenetic positions of *Clostridium chauvoei* and *Clostridium septicum* based on 16S rRNA gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, *46*(4), 1174–1176. https://doi.org/10.1099/00207713-46-4-1174

Liesener, K., Curtui, V., Dietrich, R., Märtlbauer, E., & Usleber, E. (2010). Mycotoxins in horse feed. *Mycotoxin Research*, *26*, 23–30. https://doi.org/10.1007/s12550-009-0037-8

Lombaert, G. A., Pellaers, P., Roscoe, V., Mankotia, M., Neil, R., & Scott, P. M. (2003). Mycotoxins in infant cereal foods from the Canadian retail market. *Food Additives & Contaminants*, *20*(5), 494–504. http://doi.org/10.1080/0265203031000094645

Los, A., Ziuzine, D., & Bourke, P. (2018). Current and future technologies for microbiological decontamination of cereal grains. *Journal of Food Science*, *83*(6), 1484-149. https://doi.org/10.1111/1750-3841.14181

Lou, X., Yu, H., Wang, X., Qi, J., Zhang, W., Wang, H., Si, G., Song, S., Huang, C., Liu, T., Zheng, W., & Fang, Z. (2019). Potential reservoirs and routes of *Cronobacter* transmission during cereal growing, processing and consumption. *Food Microbiology*, *79*, 90–95. https://doi.org/10.1016/j.fm.2018.12.004

Mallmann, C. A., Tyska, D., Almeida, C. A. A., Oliveira, M. S., & Gressler, L. T. (2020). Mycotoxicological monitoring of breakfast and infant cereals marketed in Brazil. *International Journal of Food Microbiology*, *331*, 108628. https://doi.org/10.1016/j.ijfoodmicro.2020.108628

Miller, R. A., Beno, S. M., Kent, D. J., Carroll, L. M., Martin, N. H., Boor, K. J., & Kovac, J. (2016). *Bacillus wiedmannii* sp. nov., a psychrotolerant and cytotoxic *Bacillus cereus* group species isolated from dairy foods and dairy environments. *International Journal of Systematic and Evolutionary Microbiology*, *66*, 4744–4753. https://doi.org/10.1099/ijsem.0.001421

Mulder, P. P. J., Pereboom-de Fauw, D. P. K. H., Hoogenboom, R. L. A. P., de Stoppelaar, J., & de Nijs, M. (2015). Tropane and ergot alkaloids in grain-based products for infants and young children in the Netherlands in 2011–2014. *Food Additives & Contaminants: Part B*, *8*(4), 284–290. https://doi.org/10.1080/19393210.2015.1089947

Muytjens, H. L., Roelofs-Willemse, H., & Jaspar, G. H. J. (1988). Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. *Journal of Clinical Microbiology*, *26*(4), 743–746. https://doi.org/10.1128/JCM.26.4.743-746.1988

Oueslati, S., Berrada, H., Mañes, J., & Juan, C. (2018). Presence of mycotoxins in Tunisian infant foods samples and subsequent risk assessment. *Food Control*, *84*, 362–369. https://doi.org/10.1016/j.foodcont.2017.08.021

Pereira, V. L., Fernandes, J. O., & Cunha, S. C. (2015). Comparative assessment of three cleanup procedures after QuEChERS extraction for determination of trichothecenes (type A and type B) in processed cereal-based baby foods by GC-MS. *Food Chemistry*, *182*, 143–149. https://doi.org/10.1016/j.foodchem.2015.01.047

Pham, P. (2021). Baby food report 2021. Statista consumer market outlook. https://www.statista.com/study/48837/baby-food-report/

Rajilić-Stojanović, M., & de Vos, W. M. (2014). The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiology Reviews*, *38*(5), 996–1047. https://doi.org/10.1111/1574-6976.12075

Reinhard, H., Rupp, H., & Zoller, O. (2008). Ergot alkaloids: Quantitation and recognition challenges. *Mycotoxin Research*, *24*, 7–13. https://doi.org/10.1007/bf02985264

Sadek, Z. I., Abdel-Rahman, M. A., Azab, M. S., Darwesh, O. M., & Hassan, M. S. (2018). Microbiological evaluation of infant foods quality and molecular detection of *Bacillus cereus* toxins relating genes. *Toxicology Reports*, *5*, 871–877. https://doi.org/10.1016/j.toxrep.2018.08.013

Scott, P. M., Zhao, W., Feng, S., & Lau, B. P. (2012). *Alternaria* toxins alternariol and alternariol monomethyl ether in grain foods in Canada. *Mycotoxin Research*, *28*, 261–6. https://doi.org/10.1007/s12550-012-0141-z

Seidler, C. (2007). *Nachweis der Fusarientoxine Deoxynivalenol und Zearalenon in Lebensmitteln* (Doctoral thesis). VVB Laufersweiler Verlag, Giessen. http://geb.uni-giessen.de/geb/volltexte/2007/4728/pdf/SeidlerCaroline-2007-04-25.pdf

Silva, J. N., Vasconcellos, L., Forsythe, S. J., de Filippis, I., & Brandão, M. L. L. (2019). Molecular and phenotypical characterization of *Cronobacter* species isolated with high occurrence from oats and linseeds. *FEMS Microbiology Letters*, *366*(1), fny289. https://doi.org/10.1093/femsle/fny289

Taylor, M. G., Amerson-Brown, M. H., Hulten, K., Cameron, L. H., Holzmann-Pazgal, G., Edwards, M. S., & Foster, C. E. (2021). Two cases of *Cronobacter sakazakii* meningitis in infants. *The Pediatric Infectious Disease Journal*, *40*(9). https://doi.org/10.1097/inf.0000000000003184

Usleber, E., Märtlbauer, E., Dietrich, R., & Terplan, G. (1991). Direct enzyme-linked immunosorbent assays for the detection of the 8-ketotrichothecene mycotoxins deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol in buffer solutions. *Journal of Agricultural and Food Chemistry*, *39*, 2091–2095. https://doi.org/10.1021/jf00011a041

Usleber, E., Renz, V., Märtlbauer, E., & Terplan, G. (1992). Studies on the application of enzyme immunoassays of the *Fusarium* mycotoxins deoxynivalenol, 3-acetyldeoxynivalenol, and zearalenone. *Journal of Veterinary Medicine Series B*, *39*, 617–627. https://doi.org/10.1111/j.1439-0450.1992.tb01213.x

Zhang, K., Flannery, B. M., Oles, C. J., & Adeuya, A. (2018). Mycotoxins in infant/toddler foods and breakfast cereals in the U.S. retail market. *Food Additives & Contaminants: Part B*, *11*, 183–190. https://doi.org/10.1080/19393210.2018.1451397

Ziver, T., Okburan, G., Akgül, Ö., Sarıbaş, S., & Kocazeybek, B. (2020). Investigation of *Cronobacter sakazakii* (*Enterobacter sakazakii*) presence in cereal infant foods. *Progress in Nutrition*, *22*, 596–602. https://doi.org/10.23751/pn.v22i2.8907

How to cite this article: Rehagel, C., Akineden, Ö., & Usleber, E. (2022). Microbiological and mycotoxicological analyses of processed cereal-based complementary foods for infants and young children from the German market. *Journal of Food Science*, 1–13. https://doi.org/10.1111/1750-3841.16106

6.3 Enzyme immunoassays for the detection of mycotoxins in plant-based milk alternatives: pitfalls and limitations

Rehagel C, Maul R, Gützkow KL, Akineden Ö

Mycotoxin Research 38, 265-274 (2022)

Author contributions:

Rehagel C: Conceptualization, formal analysis, investigation, writing - original draft preparation, writing - review and editing, validation, visualization. **Maul R**: Formal analysis, investigation, writing - original draft preparation, supervision. **Gützkow KL**: Formal analysis, investigation, writing - original draft preparation, visualization. **Akineden Ö**: Writing - review and editing, supervision, project administration

Mycotoxin Research
https://doi.org/10.1007/s12550-022-00467-x

ORIGINAL ARTICLE

Enzyme immunoassays for the detection of mycotoxins in plant-based milk alternatives: pitfalls and limitations

Christina Rehagel[1] · Ronald Maul[2] · Kim Lara Gützkow[2] · Ömer Akineden[1]

Received: 22 July 2022 / Revised: 15 August 2022 / Accepted: 16 August 2022

Abstract

Plant-based milk alternatives (PBMAs) are a potential source of mycotoxin uptake. To ensure food safety, simple and rapid testing methods of PBMAs for mycotoxins are therefore required. This study investigated the applicability of enzyme immunoassay (EIA) methods for direct testing of PBMAs without sample extraction. Mycotoxin analyses included aflatoxin B_1 (AFB_1), sterigmatocystin (STC), ochratoxin A (OTA), deoxynivalenol (DON), and T-2/HT-2-toxin (T-2/HT-2). It was found that the PBMA matrix negatively affected the EIA to varying degrees, thus affecting the reliability of the results. A dilution of PBMAs of at least 1:8 was necessary to overcome matrix interference. This resulted in calculated detection limits of 0.4 µg/L (AFB_1), 2 µg/L (STC), 0.08 µg/L (OTA), 16 µg/L (DON), and 0.4 µg/L (T-2/HT-2). After analysis of 54 PBMA products from German retail stores, positive results in at least one test system were obtained for 23 samples. However, most positive results were near the calculated detection limit. Control analyses of selected samples by LC–MS/MS for AFB_1, STC, and OTA qualitatively confirmed the presence of trace amounts of STC in some samples, but quantitative agreement was poor. It was concluded that the high diversity of ingredients used in PBMAs led to a highly variable degree of sample matrix interference even in a 1:8 dilution. Since the use of higher dilutions conflicts with the need to achieve low detection limits, the application of EIA for routine mycotoxin analysis in PBMA for mycotoxins requires further study on the development of a feasible sample preparation method.

Keywords Mycotoxins · Plant-based milk alternatives · Immunoassay · Matrix interferences

Introduction

The consumption of plant-based milk alternatives (PBMAs) has increased in Germany and other industrialised countries around the world in recent years. In addition to being "vegan", these products are commonly advertised with claims regarding health, animal welfare, and sustainable agriculture. (Janssen et al. 2016). Persistence Market Research (PMR) reported that the global market for PBMAs is currently estimated at US$ 12.1 billion and is expected to reach US$ 29.5 billion by 2031, growing with a compound annual growth rate of 9.5% (PMR 2021). In 2020, the revenue for PBMAs in Germany was US$ 452 million, which corresponds to a total consumption of around 250 million km (Statista 2021). Further forecasts showed that German consumption of PBMAs will increase to nearly 535 million km by 2026 (Statista 2021). Considering this rapidly increasing consumption, it is of great importance to ensure the food safety of these products. However, PBMAs are not specifically addressed by European Union regulation (EC) No. 1881/2006 which lays down maximum levels (MLs) for mycotoxins (EC 2006).

PBMAs presently available from the German market are an aqueous slurry of various plant materials; the main ingredients are cereals, pseudo cereals, legumes, nuts and seeds, but some also contain sugar, cocoa, or edible oil. Some products additionally contain additives (stabilisers, emulsifiers) and flavours (McClements et al. 2019; Sethi et al. 2016).

In addition to control of raw materials, a rapid and sensitive system of analysis for finished PBMAs is required

✉ Christina Rehagel
Christina.Rehagel@vetmed.uni-giessen.de

[1] Dairy Sciences, Institute of Veterinary Food Science, Justus-Liebig-University Giessen, Ludwigstrasse 21, 35390 Giessen, Germany

[2] Department Safety and Quality of Milk and Fish Products, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Hermann-Weigmann-Strasse 1, 24103 Kiel, Germany

Published online: 02 September 2022

to verify the safety of such products. Published surveys on contaminants in PBMAs in general are scarce. All contaminants typical for the raw products, i.e. heavy metals or environmental and natural toxins in general, need to be considered also for PBMAs. So far, there are only a few published studies, all with very limited sample size, which report investigations of PBMAs for mycotoxins. Although no study specific for the German market exists, data from other European countries clearly demonstrate that PBMAs may be contaminated by multiple mycotoxins belonging to different chemical groups including trichothecenes and aflatoxins (Arroyo-Manzanares et al. 2019; Hamed et al. 2017, 2019; Juan et al. 2022; Miró-Abella et al. 2017).

Published studies on the occurrence of mycotoxins in PBMAs exclusively utilised liquid chromatography coupled with either tandem mass spectrometry or fluorescence detection. While these methods are convenient in a laboratory environment, they are less suitable for rapid on-site quality control at the production site. For liquid food materials, EIA appear to be a suitable tool for rapid on-site testing and have been used for decades for the analysis of aflatoxin M_1 in cow's milk (Pecorelli et al. 2020). Therefore, this study aimed at exploring the possibility to employ a set of in-house EIA methods for different mycotoxins, analysing PBMAs directly without any sample preparation.

Materials and methods

Chemicals and reagents

Mycotoxin standards of OTA, AFB_1, STC, and DON were obtained from Sigma-Aldrich (Taufkirchen, Germany); T-2 toxin was from Biopure (Tulln, Austria). After dissolving the mycotoxin standards in methanol (OTA, AFB_1, DON, T-2) or acetonitrile (STC), the concentration and purity of all stock solutions (except T-2) were checked by UV spectroscopy (Shimadzu, Duisburg, Germany), using published data (Cole and Schweikert 2003; Cole et al. 2003) for comparison and calculation. ^{13}C-labelled standard solutions for AFB_1, STC, and OTA were obtained from Biopure™ (Romer Labs Deutschland GmbH, Butzbach, Germany). All other reagents and chemicals used were at least of analytical grade. Methanol (LC–MS grade) and acetonitrile (ACN) (LC–MS grade) were purchased from Supelco® (Merck KGaA, Darmstadt, Germany). n-Heptane, dimethylsulfoxide (DMSO), ACN (HPLC grade), and anhydrous magnesium sulphate ($MgSO_4$) were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany), while ammonium formate (NH_4COOH) and acetic acid (HAc) were from Merck KGaA (Darmstadt, Germany). Formic acid (FA) was from VWR International GmbH (Darmstadt, Germany). Ultrapure water was obtained through the use of a water purification device (PURELAB flex 3, ELGA LabWater, Veolia Water Technologies Deutschland GmbH, Celle Germany). AflaTest $_{WB}$ SR^+ immunoaffinity columns (IAC) were purchased from VICAM (Milford, USA) and contained monoclonal antibodies that specifically bind aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2) and STC. According to the manufacturer, the column capacity was 1000 ng for total aflatoxins and recovery for B_1, B_2, G_1, and G_2 was ≥ 90% for spiking level of 2 ng and 500 ng.

Sample materials

A total of 54 samples of various PBMA products, with the majority ($n = 34$) of these labelled as of "organic produce", were purchased from retail shops and specialised "organic food" stores in the area of Giessen, Hesse, Germany, in 2020. All products were purchased as offered, in original packaging. According to product information, the products originated from 17 different manufacturers from eight countries in Europe; the majority was from German producers ($n = 28$). The main ingredients were water and vegetable raw materials of a content ranging from 8.7 to 17% for cereal-based or pseudocereal-based PBMA (oat, rice, spelt, millet, buckwheat), 2.3–8.4% for nut-based products (hazelnut, almond, coconut, cashew), 4–10% for products based on legumes (soy bean, pea, lupine), 3% for hemp-based products, and 4.9–21% for PBMA consisting of ingredient mixtures (oat + almond, rice + almond, rice + coconut, rice + coconut + cashew). In addition, a few products contained minor amounts of sunflower or rapeseed oil, cocoa, sugar, and salt. Most of the PBMAs without claims of organic produce contained stabilisers and emulsifiers. All products were heat-treated, mostly by ultra-high temperature treatment (> 135 °C); a few were pasteurised. The remaining shelf life of ultra-high temperature-treated PBMA products was > 4 months and for pasteurised PBMA products > 2 weeks at the time of purchase.

EIA analysis

Sample pretreatment

Before opening, each package of PBMA was manually shaken to mobilise sedimented particles. Then, a portion for follow-up analyses of about 50 mL was transferred into plastic test tubes and frozen at − 18 °C. Material from products containing stabilisers or emulsifiers was centrifuged (3000 × g, 10 min, 20 °C). Then, sample material was diluted with EIA buffer solution as required for each test system, and dilutions ranging from 1:2 to 1:20. For AFB_1, T-2/HT-2, and STC analysis, sample dilutions and toxin standard curves were prepared in phosphate buffered saline (PBS; 0.01 mol/L; pH 7.2) containing 10% methanol. For DON analysis, samples were diluted in PBS (pH 7.2). For

OTA analysis, samples were diluted with aqueous $NaHCO_3$ solution (0.13 mol/L).

Effect of sample matrix on EIA standard curve

Since no certified toxin-negative PBMA material was available, the extent of sample matrix interference was assessed by comparing toxin standard curves made in EIA buffer solution with toxin standard curves made with diluted PBMA. For this series of experiments, one sample each from every major product group was selected. The minimal dilution which yielded standard being congruent with the buffer solution standard curve was then used for analyses of the remaining sample materials.

Analysis of artificially contaminated sample material

As an additional quality control, six PBMA materials were artificially contaminated with the mycotoxins under study by adding 20–100 µL of toxin standard solution per millilitre of sample at appropriate concentrations (OTA 0.2–0.8 µg/L; AFB_1, T-2/HT-2 1–4 µg/L; STC 4–16 µg/L; DON 30–120 µg/L). Four replicates of all standard and sample solutions were analysed, and each PBMA sample was analysed in a single dilution.

EIA test procedure

For mycotoxin analysis of PBMA samples, EIA were performed using microtiter plates (MaxiSorp, Nunc, Roskilde, Denmark) as described earlier for AFB_1 (Gathumbi et al. 2001), STC (Wegner et al. 2016), OTA (Schneider et al. 2001), DON (Curtui et al. 2003), and T-2/HT-2 (Esgin et al. 1989). All EIA were performed based on competitive direct test format, using the double antibody method for DON and T-2/HT-2. The EIA absorbance values at 450 nm were measured using a microplate reader (Tecan Sunrise, Crailsheim, Germany) and evaluated by Magellan calculation software (Tecan, Crailsheim, Germany). EIA absorbance values of standard concentrations were normalised by dividing the mean absorbance values of the standard or diluted sample solution (B) by the absorbance value of the blank (B_0) and then multiplying by 100 ($B/B_0 \times 100$).

Control analyses for AFB_1, STC, and OTA by LC–MS/MS

Sample pretreatment

For the control analyses, five samples which had yielded highly positive results in EIA were selected for LC–MS/MS analysis for AFB_1, STC, and OTA. Four of these samples (MA11, MA25, MA31, MA54) contained cocoa in addition to their main ingredient, and one sample contained black rice (MA48). For these series of experiments, extracts for AFB_1 and STC analyses were prepared by liquid–liquid partitioning (LLP) of a 10-mL test portion twice with each 40 mL of ethyl acetate. The two organic phases from each sample were collected and combined, the solvent removed in a rotary evaporator at 50 °C, then the residue dissolved with 10 mL of methanol. One millilitre of the extract was transferred to a conical flask and evaporated at 50 °C in a rotary evaporator. The residue was dissolved in 2 mL of PBS containing 10% methanol and analysed by EIA. The calculated limit of detection (LOD) in LLP extracts was 0.1 µg/L for AFB_1 and 0.2 µg/L for STC, respectively.

Further purification of the LLP extracts was done using IAC columns. A 5-mL portion of the LLP extract was diluted with 20 mL PBS (pH 7.2), solid particles removed by centrifugation (3000 × g, 10 min, 20 °C), then the supernatant was filtered through a paper filter. The filtered solution was passed through an IAC column, following the manufacturers' instructions. Toxins were eluted from the column with two, and consecutively added 1.5-mL portions of methanol. The methanolic eluate was collected in a conical flask and 1 mL was used for LC–MS/MS analysis. The remaining solvent (2 mL) was removed in a rotary evaporator at 50 °C and the residue dissolved with 2 mL of 10% methanol/PBS for EIA analysis. Based on the cut-off value of the EIA standard curves, the calculated LOD for IAC extracts was 0.04 µg/L for AFB_1 and 0.06 µg/L for STC, respectively.

LC–MS/MS analysis

The analysis was performed on a 1290 Infinity II LC system (Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany). Analytes were separated on a Gemini reversed phase C18 analytical column, 100 × 3.0 mm, 5.0 µm (Phenomenex®, Aschaffenburg, Germany), at an oven temperature of 35 °C, while the injection volume was 4 µL. LC separation was performed using a gradient elution of water (with 0.1% formic acid, 300 mg/L ammonium formate) and methanol (with 0.1% formic acid, 300 mg/L ammonium formate) and a flow rate of 0.5 mL/min. The gradient programme started at 5% organic solvent for 0.8 min, raising to 50% by minute 1.5, to 55% by minute 2.5, to 70% by minute 5.5, to 76 by minute 6.5, and to 95% by minute 15.5. Starting from minute 17.0, the organic percentage reverted to the starting conditions of 5% by minute 17.5 and was kept until the end of the run at 19.5 min. MS detection was conducted using a triple quadrupole MS (QTRAP 6500 +, Sciex Germany GmbH, Darmstadt, Germany) operating in both positive and negative electro spray ionisation (ESI) mode and measuring in multiple reaction mode (MRM) with the following settings: curtain gas 40, collision gas medium, temperature 350 °C, the ± ion spray voltage 4500 V, nebuliser

gas flow of 50, heater gas flow of 45, and dwell time varied. The analytical parameters for AFB_1, STC, and OTA are shown in Table 1.

For sample preparation, 62 µL of the IS mixture was added to 1 mL of the PBMA sample, and the samples were extracted with 938 µL of ACN containing 0.1% FA. After shaking for 10 min (IKA-VIBRAX VXR, IKA®-Werke GmbH & CO. KG, Staufen, Germany), 0.1 g NaCl and 0.4 g $MgSO_4$ were added and shaken for another 5 min. The samples were centrifuged at 10,000 × g for 7 min at room temperature (Avanti JXN-30, Beckman Coulter GmbH, Krefeld, Germany). A total of 0.8 mL of supernatant was transferred in a tube and 0.8 mL of n-heptane added. After shaking for 5 min (IKA-VIBRAX_VXR, IKA®-Werke GmbH & CO. KG, Staufen, Germany), the n-heptane phase was discarded. ACN phase was transferred into a 2-mL reagent tube containing 100 µL DMSO as keeper solvent and the ACN was evaporated until only the DMSO proportion remained (Concentrator plus, Eppendorf AG, Hamburg, Germany). A total of 200 µL ACN with 0.1% FA were added to the residual liquid and vortexed. After additional sonicating for 10 min (Transsonic 460, Elma Schmidbauer GmbH, Singen, Germany), 300 µL of H_2O was added and the samples were sonicated again for 10 min and vortexed afterwards. The extracts were filtered using a regenerated cellulose 0.45-µm syringe filter unit (ProSense B.V., Munich, Germany). The LLP and IAC extracts were diluted 1/1 with water before the LC–MS/MS analysis.

Results and discussion

Given their high sensitivity, EIA seem to be a convenient tool for mycotoxin testing in liquid sample materials such as PBMA. However, it was observed that the highly variable composition of PBMAs and their high content of non-soluble material exerted the matrix influence which did effect each individual test system to varying degrees. This study was also impeded by the fact that no defined reference material, either mycotoxin-free or with certified mycotoxin content, is available for PBMA or comparable matrices. Therefore, we first subjected a larger number ($n = 54$) of PBMA products to EIA analyses at different dilutions with buffer solution. Selected materials from each major group of products were then used to establish toxin standard curves in matrix (Fig. 1). PBMAs up to a dilution of 1:4 yielded strongly left-shifted standard curves with depressed B_0 values, indicating that false-positives and overestimation of the toxin content were major issues. Except for the STC-EIA, toxin standards prepared in 1:8 diluted PBMA matrix resulted in standard curves which were nearly identical with the buffer solution standard curve, observed for all different matrices. Therefore, a minimum dilution factor of 8 was applied for all subsequent analyses. In the STC-EIA, an even higher dilution (1:20) was required to eliminate left-shifted standard curves. The necessity to dilute PBMAs for EIA analyses negatively affected the achievable, calculated detection limit in sample matrix. The LOD summarised in Table 2 were considered to be still in a relevant concentration range for DON and T-2/HT-2 while for AFB_1, STC, and OTA, they were probably insufficient.

Adding toxin standard solution to PBMA material before dilution yielded results which were still quite variable, depending on both type of matrix and spiking level (Table 3). This indicates that even at a 1:8 dilution (STC-EIA: 1:20), some remaining matrix interference could cause up to 50% deviation from the nominal value. A possible reason for these matrix interferences are the proteins contained in PBMAs. For example, Wang et al. (2015) investigated the influence of fish proteins on competitive indirect EIA and demonstrated that fish proteins interfere with immunological reactions by binding to both primary antibodies and enzyme-labelled secondary antibodies.

When the EIA results of mycotoxin analysis for all 54 samples were grouped according to the main ingredients

Table 1 Analytical parameters of quantitative determination of the analytes and their isotopically labelled internal standards with the HPLC–MS/MS; ESI (+) mode; multiple reaction monitoring mode; the second product ion was used as a qualifier for the confirmation of identity for each analyte; for all analytes, compound optimisation with the LC–MS was performed

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)	DP[a] (V)	CE[b] (eV)	CXP[c] (V)
AFB_1	313.0	285.0	111	33	16
		241.0	111	49	26
^{13}C-Aflatoxin B_1	330.0	301.0	111	33	16
STC	325.0	310.0	96	33	18
		281.0	96	49	32
^{13}C-Sterigmatocystin	343.0	297.0	96	49	32
OTA	404.0	239.1	31	29	14
		357.9	31	19	24
^{13}C-Ochratoxin A	424.1	250.1	31	29	14

[a] *DP* declustering potential

[b] *CE* collisions energy

[c] *CXP* collision cell exit potential

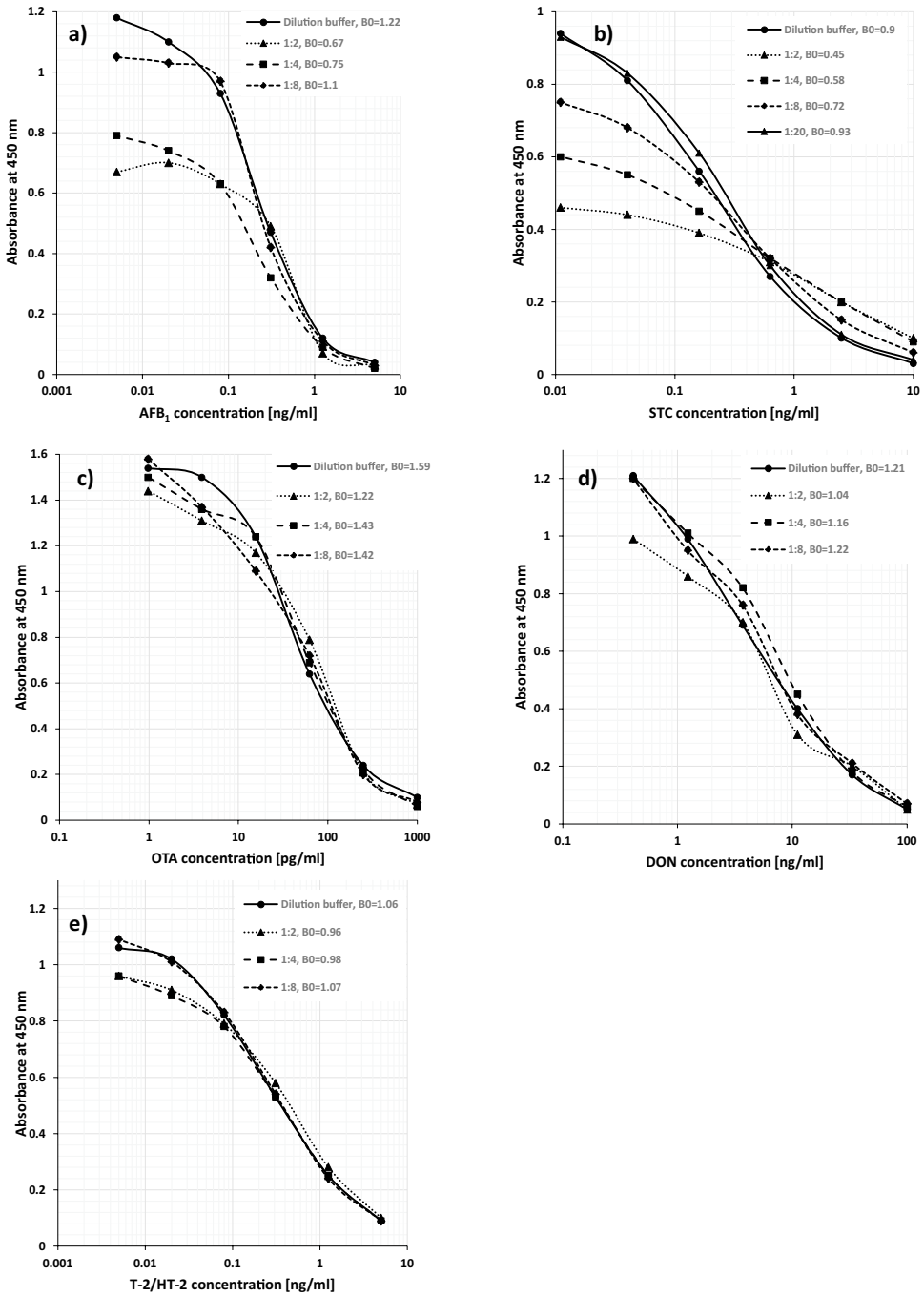

Fig. 1 Example of PBMA sample matrix effects on EIA toxin standard curves (only oat-based PBMA shown), indicating the absorbance value of the blank (B_0) of each standard curve. **a)** AFB_1: standard curves made in matrix at dilutions with test buffer of 1:2 and 1:4 resulted in a depression of the absorbance at 450 nm. At a dilution of 1:8, the standard curve was almost fully congruent with the standard curve in buffer solution. **b)** STC: strong absorbance signal depression for standard curves made in matrix at dilutions of up to 1:8, at a 1:20 dilution, standard curve which was almost congruent with standard curve in buffer solution. **c)** OTA. **d)** DON. **e)** T-2/HT-2. Each of the six standard curve data points represents the mean absorbance at 450 nm of four replicate wells. The coefficients of variation ranged from 3 to 10% and did not differ between all curves

(Table 4), it became clear that the EIA for DON and T-2/HT-2 in general yielded results which were plausible in view of the trichothecene frequency in cereals. However, with regard to T-2/HT-2, there were two exceptions: one soy-based sample and one hemp-based sample showing a weak positive result for T-2/HT-2. The fact that no studies on the occurrence of mycotoxins in hemp seeds are currently available makes a plausibility assessment difficult in this case. Even though the occurrence of T-2/HT-2 in soy is not common, it cannot be completely excluded. Other study results show that, in addition to cereals susceptible to T-2/HT-2, these toxins can also occur in soybean from Argentina (Barros et al. 2011). With these two exceptions, trichothecene mycotoxins were detected in cereal-containing PBMAs only. The levels measured for these samples corresponded well with contamination data for oats specifically (EFSA 2017; Curtui et al. 2009), and for cereals in general (Gottschalk et al. 2009). Furthermore, they are in good agreement with the results reported by Miró-Abella et al. (2017). Considering that the total amount of solids in these products typically ranged from 5 to 10%, the

Table 2 Cross-reactions and standard curve parameters of the mycotoxin EIA and calculated detection limit in PBMA

Test system	Known relevant cross-reactions	Standard curve		Minimum sample dilution factor for EIA analysis	Calculated detection limit in PBMA µg/L
		IC_{50} value, mean* ± SD, µg/L	Cut-off value (IC_{20}), mean* ± SD, µg/L		
AFB_1	$AFB_{1/2}$, $AFG_{1/2}$, AFM_1, AFB2a, AFG2a, AFP_1, AFQ_1, Aflatoxicol (Gathumbi et al. 2001)	0.14 ± 0.02	0.05 ± 0.01	8	0.4
STC	*O*-methylsterigmatocystin (Wegner et al. 2016)	0.29 ± 0.09	0.08 ± 0.02	20	2
OTA	OTA, OTB (Schneider et al. 2001)	0.04 ± 0.007	0.01 ± 0.004	8	0.08
DON	DON and its 8-ketotrichothecene analogues (Curtui et al. 2003)	5.15 ± 0.92	1.67 ± 0.48	8	16
T-2/HT-2	T-2, HT-2 (Esgin et al. 1989)	0.23 ± 0.04	0.05 ± 0.01	8	0.4

*16–27 plates performed for each test in a period of 12 months, except T-2/HT-2 (5 tests)

concentration of these toxins in the cereal ingredients should be about 10–20-fold higher, in a range roughly between 100 and 800 µg/kg for DON, and between 4 and 80 µg/kg for T-2/HT-2. This would be well within the range of reported data for these toxins in European oat.

The AFB_1-EIA showed a positive result for AFB_1 in one sample based on black whole grain rice, in addition to three pea- or oat-based products with cocoa. On the other hand, almonds are known to potentially contain aflatoxins (Kanik and Kabak 2019), but the AFB_1-EIA gave negative results for this group of products. The reason for these findings could be that the LOD for this toxin in these matrices did not allow sufficiently sensitive analysis. Assuming that the raw materials complied with European Union regulation 1881/2006, the aflatoxin levels which could be expected in PBMA based on soy or almonds would probably be below 0.4 µg/L, which is the calculated LOD of the AFB_1-EIA. A similar situation was observed for the STC-EIA (LOD 2 µg/L), which gave positive results in just one soy-based PBMA and in two oat-based products. The few positive results in the OTA-EIA (LOD 0.08 µg/L) for PBMA based on soy or oat were found for the same samples. All these three products contained cocoa, in addition to the main ingredient. Furthermore, a weakly positive result for OTA was found in the sample based on black whole grain rice.

Further work on elucidation of matrix effects therefore focussed on products containing cocoa as an ingredient and the product based on black whole grain rice. In fact,

Table 3 EIA results for four different PBMA product groups, artificially contaminated with mycotoxins. For each concentration level, one sample from each category was spiked

Test system	Toxin added, µg/L	Toxin found, in % of added amount					
		Soy	Oat	Almond	Coconut	Mean	RSD[a], %
AFB_1	1	80	133	119	83	104	26
	2	90	86	133	128	109	25
	4	97	83	88	156	106	34
STC	4	123	89	148	107	117	25
	8	115	92	142	94	111	23
	16	127	89	142	92	113	26
OTA	0.2	146	85	84	61	94	36
	0.4	114	75	77	69	84	20
	0.8	124	89	91	83	97	18
DON	30	76	104	110	65	89	22
	60	89	133	117	100	110	19
	120	88	129	113	94	106	19
T-2/HT-2	1	64	103	84	63	79	19
	2	79	101	64	66	78	17
	4	87	65	57	56	66	14

[a]Relative standard deviation

Table 4 EIA results for four categories of PBMA ($n = 54$)

		Soy ($n = 7$)	Almond ($n = 7$)	Oat ($n = 14$)	Single[a] and mixed[b] ingredients ($n = 26$)
AFB_1	*n* positive/*n*	0/7	0/7	2/14	2/26
	Range	-	-	-	0.6–0.8
STC	*n* positive/*n*	1/7	0/7	2/14	0/26
	Range	-	-	-	-
OTA	*n* positive/*n*	1/7	0/7	2/14	1/26
	Range	-	-	0.2–0.4	-
DON	*n* positive/*n*	0/7	0/7	3/14	2/26
	Range	-	-	16–22	17–43
T-2/HT-2	*n* positive/*n*	1/7	0/7	12/14	8/26
	Range	-	-	0.4–4	0.4–1

[a]Spelt, millet, rice, buckwheat, hazelnut, cashew, pea (with/without cocoa), lupin, hemp

[b]Oat + almond, rice + almond, rice + coconut, rice + coconut + cashew

these products yielded the highest results in the AFB_1-EIA, STC-EIA, or OTA-EIA. Two products were oat-based, one was based on soy, and another was based on peas, but all contained cocoa according to package labels. Although cocoa is known to be susceptible to aflatoxins and OTA contamination (Copetti et al. 2011; Gilmour and Lindblom 2008; Raters and Matissek 2003), it seemed unlikely that the high levels in the EIA were caused by the low percentage of cocoa (< 1.5%) in the product; for this reason, these samples were additionally analysed by LC–MS/MS. In an initial attempt to improve the detection limit by lowering the sample dilution factor, extracts were prepared by LLP of these samples with ethyl acetate, followed by a further clean-up step on IAC columns. The results of this comparison analysis (Table 5) showed virtually no agreement between EIA and LC–MS/MS. Furthermore, the EIA results for diluted sample and sample extracted by LLP or IAC also gave fully inconsistent results. LLP extracts were

Table 5 Comparison of EIA and LC–MS/MS results for AFB_1, STC, and OTA in diluted sample, in extracts after liquid–liquid partitioning (LLP), and in LLP extracts plus IAC clean-up for five selected PBMA samples

Sample no.	Sample description	AFB_1, µg/L		STC, µg/L		OTA, µg/L	
		EIA	LC–MS/MS	EIA	LC–MS/MS	EIA	LC–MS/MS
MA11	Sample (soy drink cocoa)	< 0.4	< 0.002	2	< 0.005	0.7	< 0.288
MA11	LLP extract	0.3	< 0.002	2	< 0.002	n.a	< 0.288
MA11	IAC extract	< 0.04	< 0.002	0.09	< 0.002	n.a	< 0.288
MA25	Sample (oat drink cocoa)	0.5	< 0.002	3	< 0.005	0.4	< 0.288
MA25	LLP extract	0.3	< 0.002	2	< 0.002	n.a	< 0.288
MA25	IAC extract	< 0.04	< 0.002	0.08	< 0.002	n.a	< 0.288
MA31	Sample (oat drink cocoa)	0.5	< 0.002	3	< 0.002	0.2	< 0.288
MA31	LLP extract	0.4	< 0.002	2	< 0.002	n.a	< 0.288
MA31	IAC extract	< 0.04	< 0.002	0.07	< 0.002	n.a	< 0.288
MA48	Sample (black whole grain rice)	0.6	< 0.002	< 2	< 0.002	0.1	< 0.288
MA48	LLP extract	0.2	< 0.002	1	< 0.002	n.a	< 0.288
MA48	IAC extract	< 0.04	< 0.002	0.1	< 0.002	n.a	< 0.288
MA54	Sample (pea drink cocoa)	0.8	< 0.002	< 2	0.06	< 0.08	< 0.288
MA54	LLP extract	0.2	< 0.002	2	< 0.002	n.a	< 0.288
MA54	IAC extract	< 0.04	< 0.002	0.2	< 0.002	n.a	< 0.288

n.a. not analysed, *LOD* limit of detection (EIA for samples: AFB_1, 0.4 µg/L; STC, 2 µg/L; OTA, 0.08 µg/L; EIA for LLP extracts: AFB_1, 0.1 µg/L; STC, 0.2 µg/L; EIA for IAC extracts: AFB_1, 0.04 µg/L; STC, 0.06 µg/L; LC–MS/MS for sample preparation as described above in LC–MS/MS analysis: AFB_1, 0.002 µg/L; STC, 0.002 µg/L; OTA, 0.288 µg/L), *LOQ* limit of quantification (LC–MS/MS for sample preparation, calculated for the conventional clean up without LLP or IAC, as described above in LC–MS/MS analysis: AFB_1, 0.008 µg/L; STC, 0.005 µg/L; OTA, 0.95 µg/L)

still positive, albeit at lower levels, in the tests for AFB_1 and STC. IAC extracts were all EIA negative for AFB_1, but still weakly positive for STC. Sample MA54, which had been negative in diluted sample material, were tested positive for STC by EIA in LLP and IAC extracts. This indicates that at least for cocoa-containing samples, the EIA are not applicable to PBMA without significant improvement of the sample preparation method. Further work will study on a broader sample matrix basis, whether similar discrepancies are to be expected for other PBMA products. The costlier

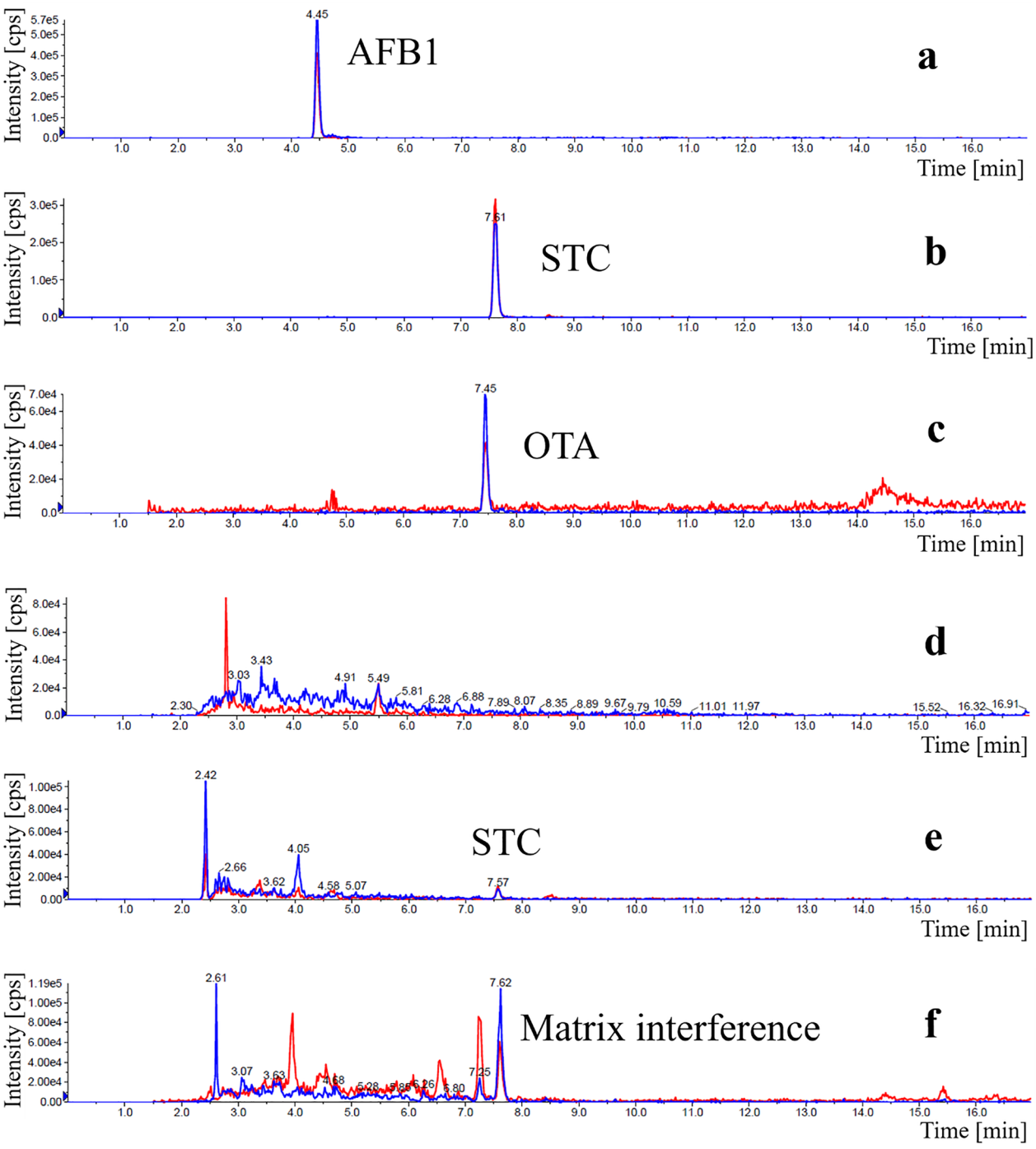

Fig. 2 LC–MS/MS chromatograms in ESI (+) mode of an AFB_1 **a**, STC **b**, and OTA **c** reference and of a STC positive soy-based PBMA sample (MA11) containing cocoa (**d**–**f**). Extract ion chromatograms in the multiple reaction monitoring mode (MRM) showing mass transitions (m/z) **a**, **d** 313.0→285.0; **b**, **e** 325.0→310.0; and **c**, **f** 404.0→239.1

and time-consuming LC–MS/MS analysis achieves lower LOQ. Thus, traces of STC were detected in the comparative LC–MS/MS analysis in some samples (Fig. 2), indicating that the further work is warranted to clarify the contamination situation. Although LC–MS/MS analysis revealed the presence of a peak showing both typical mass transitions for OTA in some PBMAs, OTA contamination could not be confirmed. Due to the small retention time shift of 0.2 min compared to the OTA standard, the peak was caused by a matrix interference (Fig. 2). Additionally, an OTA adduct can be out-ruled as the mass spectrum does not show the typical pattern for a chlorine-containing compound (data not shown).

Our data suggest that there is the possibility of a mycotoxin contamination in PBMA that can contribute to the overall mycotoxin exposure. This finding might be of interest for consumer groups that consume particularly high amounts of these drinks. However, currently, there are no PBMA consumption data available for Germany. Thus, an estimation of the contribution to the overall exposure is not feasible at this point.

In conclusion, this study showed that the PBMA matrix is highly complex and presents a challenge for EIA methods, although not all test systems were found to be equally susceptible to matrix interference. In any case, careful study of the effectiveness of sample treatment is required for each EIA and should be followed by broad validation studies. Before EIA could be recommended for general routine screening of PBMAs, such studies should include all relevant varieties of composition and all product groups. Unlike milk, analysis of PBMA after dilution with buffer has a high risk of false-positive or false-negative results.

Acknowledgements We thank Margit Kessler and Renate Stumpf for their excellent technical assistance.

Author contribution Conceptualisation: Christina Rehagel; formal analysis and investigation: Christina Rehagel, Ronald Maul, Kim Lara Gützkow; writing—original draft preparation: Christina Rehagel, Ronald Maul, Kim Lara Gützkow; writing—review and editing: Christina Rehagel; Ömer Akineden; validation: Christina Rehagel; visualisation: Christina Rehagel, Kim Lara Gützkow; supervision: Ömer Akineden, Ronald Maul; project administration: Ömer Akineden.

Declarations

Conflict of interest All authors declare no competing interests.

References

Arroyo-Manzanares N, Hamed AM, García-Campaña AM, Gámiz-Gracia L (2019) Plant-based milks: unexplored source of emerging mycotoxins. A proposal for the control of enniatins and beauvericin using UHPLC-MS/MS. Food Addit Contam Part B 12:296–302. https://doi.org/10.1080/19393210.2019.1663276

Barros G, García D, Oviedo M, Ramirez M, Torres A, Lattanzio V, Pascale M, Chulze S (2011) Survey of T-2 and HT-2 toxins in soybean and soy meal from Argentina using immunoaffinity clean-up and high performance liquid chromatography. World Mycotoxin J 4:189–197. https://doi.org/10.3920/WMJ2010.1272

Cole RJ, Schweikert MA (2003) Handbook of secondary fungal metabolites, vol 1. Academic Press, New York

Cole RJ, Jarvis BB, Schweikert MA (2003) Handbook of secondary fungal metabolites, vol 3. Academic Press, New York

Copetti MV, Iamanaka BT, Pereira JL, Fungaro MH, Taniwaki MH (2011) Aflatoxigenic fungi and aflatoxin in cocoa. Int J Food Microbiol 148:141–144. https://doi.org/10.1016/j.ijfoodmicro.2011.05.020

Curtui V, Seidler C, Dietrich R, Märtlbauer E, Schneider E, Usleber E (2003) Bestimmung von Deoxynivalenol in Brot und Bier. Mycotoxin Res 19:144–148

Curtui V, Usleber E, Trebstein A, Lauber U, Hocke K, Dietrich R, Märtlbauer E, Majerus P, Zimmer M, Klaffke H, Gareis M, Betsche T, Langenkämper G (2009) Improvement and validation of analytical methods for type A trichothecenes (T-2 toxin and HT-2 toxin) and occurrence of these mycotoxins in foods from the German market. Bundesanstalt für Landwirtschaft und Ernährung (BLE). Available from: https://service.ble.de/ptdb/index2.php?detail_id=88841&site_key=145&stichw=trichothecene&zeilenzahl_zaehler=4#newContent. (Accessed on 04 Jul 2022)

EC - European Commission (2006) Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union L 364/5. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:EN:PDF. (Accessed on 26 Jan 2022)

EFSA - European Food Safety Authority (2017) Human and animal dietary exposure to T-2 and HT-2 toxin. EFSA J 15(8):4972. https://doi.org/10.2903/j.efsa.2017.4972

Esgin S, Märtlbauer E, Terplan G (1989) Entwicklung und Anwendung eines enzymimmunologischen Verfahrens zum Nachweis von T-2 Toxin in Milch. Arch Lebensm Hyg 40:109–112

Gathumbi JK, Usleber E, Märtlbauer E (2001) Production of ultrasensitive antibodies against aflatoxin B_1. Lett Appl Microbiol 32:349–351. https://doi.org/10.1046/j.1472-765X.2001.00914.x

Gilmour M, Lindblom M (2008) Management of ochratoxin A in the cocoa supply chain: a summary of work by the CAOBISCO/ECA/FCC working group on ochratoxin A. In: Leslie JF, Bandyopadhyay R, Visconti A (eds) Mycotoxins: Detection Methods. Management, Public Health and Agricultural Trade, CABI, Wallingford, pp 231–243

Gottschalk C, Barthel J, Engelhardt G, Bauer J, Meyer K (2009) Simultaneous determination of type A, B and D trichothecenes and their occurrence in cereals and cereal products. Food Addit Contam 26:1273–1289. https://doi.org/10.1080/02652030903013260

Hamed AM, Arroyo-Manzanares N, García-Campaña AM, Gámiz-Gracia L (2017) Determination of Fusarium toxins in functional

vegetable milks applying salting out assisted liquid-liquid extraction combined with ultra-high performance liquid chromatography tandem mass spectrometry. Food Addit Contam Part A 34:2033–2041. https://doi.org/10.1080/19440049.2017.1368722

Hamed AM, Abdel-Hamid M, Gámiz-Gracia L, García-Campaña AM, Arroyo-Manzanares N (2019) Determination of aflatoxins in plant-based milk and dairy products by dispersive liquid-liquid microextraction and high-performance liquid chromatography with fluorescence detection. Anal Lett 52:363–372. https://doi.org/10.1080/00032719.2018.1467434

Janssen M, Busch C, Rödiger M, Hamm U (2016) Motives of consumers following a vegan diet and their attitudes towards animal agriculture. Appetite 105:643–651. https://doi.org/10.1016/j.appet.2016.06.039

Juan C, Mañes J, Juan-García A, Moltó JC (2022) Multimycotoxin analysis in oat, rice, almond and soy beverages by liquid chromatography-tandem mass spectrometry. Appl Sci 12:3942. https://doi.org/10.3390/app12083942

Kanik T, Kabak B (2019) Aflatoxins in almonds: monitoring and exposure assessment. J Food Saf 39:e12646. https://doi.org/10.1111/jfs.12646

McClements DJ, Newman E, McClements IF (2019) Plant-based milks: a review of the science underpinning their design, fabrication, and performance. Compr Rev Food Sci Food Saf 18:2047–2067. https://doi.org/10.1111/1541-4337.12505

Miró-Abella E, Herrero P, Canela N, Arola L, Borrull F, Ras R, Fontanals N (2017) Determination of mycotoxins in plant-based beverages using QuEChERs and liquid chromatography-tandem mass spectrometry. Food Chem 229:336–372. https://doi.org/10.1016/j.foodchem.2017.02.078

Pecorelli I, Guarducci N, von Holst C, Bibi R, Pascale M, Ciasca B, Logrieco AF, Lattanzio VMT (2020) Critical comparison of analytical performances of two immunoassay methods for rapid detection of aflatoxin M_1 in milk. Toxins 12:270. https://doi.org/10.3390/toxins12040270

PMR - Persistence Market Research (2021) Plant-based milk market outlook (2021–2031). Available online: https://www.persistencemarketresearch.com/market-research/plant-based-milk-market.asp. (Accessed on 03 May 2022)

Raters M, Matissek R (2003) Neue Studien zur Analytik und zum Vorkommen von Ochratoxin A in Kakao und kakaohaltigen Erzeugnissen Abschlussbericht Projektzeitraum: 01.07.2000–31.12.2002 Projekt Nr. 33 der Stiftung der Deutschen Kakao- und Schokoladenwirtschaft. Available online: http://www.yumpu.com/de/document/view/5973570/neue-studien-zur-analytik-und-zum-vorkommen-von-ochratoxin-a-in-. (Accessed on 12 May 2022)

Schneider E, Usleber E, Dietrich R, Märtlbauer E (2001) Entwicklung eines hochempfindlichen Enzymimmuntests zum Nachweis von Ochratoxin A. Mycotoxin Res 17A:170–173

Sethi S, Tyagi SK, Anurag RK (2016) Plant-based milk alternatives an emerging segment of functional beverages: a review. J Food Sci Technol 53:3408–3423. https://doi.org/10.1007/s13197-016-2328-3

Statista (2021) Statista consumer market outlook. Total consumption of milk substitutes in Germany from 2013 to 2026. Available online: https://www.statista.com/forecasts/1277954/milk-substitute-germany-consumption. (Accessed on 03 May 2022)

Wang X, Lin H, Cao L, Zhang X, Sui J (2015) The matrix interference to the immunoassay of food samples: the effect of some proteins in aquatic products. Food Agr Immunol 27:230–241. https://doi.org/10.1080/09540105.2015.1086317

Wegner S, Bauer JI, Dietrich R, Märtlbauer E, Usleber E, Gottschalk C, Gross M (2016) A highly specific competitive direct enzyme immunoassay for sterigmatocystin as a tool for rapid immunochemotaxonomic differentiation of mycotoxigenic *Aspergillus* species. Lett Appl Microbiol 64:124–130. https://doi.org/10.1111/lam.12702

7 Appendix

7.1 Summary

Indole alkaloids have already been detected in a number of foods and beverages in widely varying concentrations. However, the data on the occurrence and detection of these compounds is still very limited. A similar picture of the data situation emerges for the mycotoxicological quality of special products such as processed cereal-based foods for infants and young children (PCF) as well as for plant-based milk alternatives (PBMAs) consumed by both adults and children. The studies conducted in this work provided new insights into the detection and occurrence of mycotoxins, especially indole diterpene alkaloids (IDTs), in foods.

The first study (**study 1**) was concerned with the development of an enzyme immunoassay (EIA) for the detection of the IDTs penitrem A (PTA) in methanolic mycelial extracts prepared from fungal cultures of mouldy food. To cover as broad a spectrum of indole-type toxins as possible, two established EIAs for the detection of paxilline (PAX) and ergoline alkaloids were included in the experiments. Analysis of the mycelium extracts by EIAs revealed that fungi derived from spoiled food produce PTA, PAX and other ergoline alkaloids. Comparative investigations using LC-MS/MS showed that in addition to PTA and PAX, their analogues (penitreme B-F; deoxy-PAX) could also be detected in mycelium extracts. This finding provided evidence that the PTA-EIA detects not only PTA but also its analogues. A further investigation of selected PTA-positive fungal isolates by sequencing the internal transcribed spacer region and the β-tubulin gene resulted in an identification of *P. crustosum* and *P. polonicum*, whereby *P. polonicum* was identified as a PTA former for the first time.

The second study (**study 2**) investigated the microbiological and mycotoxicological quality of PCF from the German market. A number of microbiological parameters (aerobic mesophilic plate count, moulds, *Enterobacteriaceae*, *Cronobacter* spp., presumptive *Bacillus cereus*) were investigated, using standard microbiological methods in each case. The mycotoxin studies included deoxynivalenol (DON), zearalenone (ZEN), alternariol (AOH), T-2/HT-2 toxin (only samples containing oats) and ergot alkaloids, with sample extraction based on established methods. **Study 2** showed the detection of low concentrations of AOH, DON, T-2/HT-2 (oat-containing products only) in PCF, whereas ZEN and ergot alkaloids could not be detected. Another important finding was that a few samples contained low numbers of opportunistic

pathogens, in particular *Cronobacter sakazakii*, *Acinetobacter* spp. and *Pantoea* spp. as well as enterotoxigenic *Bacillus wiedmannii*. The results obtained within the study were all within the existing European Union regulations or international guidelines.

Study 3 investigated the applicability of mycotoxin-EIAs for direct analysis of PBMAs of different composition without prior sample extraction. For this purpose, the extent of matrix influence of different PBMA matrices on the EIAs for the detection of aflatoxin B_1 (AFB_1), sterigmatocystin (STC), Ochratoxin A (OTA), DON and T-2/HT-2 was first determined. It was found that the matrix influence turned out to be test-specific and consequently different high dilutions of the samples in the respective EIAs were necessary. After analysis of the PBMA samples from the German market, positive results were obtained in at least one test system for 43 % of the samples, with most positive results being close to the calculated detection limit. Concomitant LC-MS/MS analyses of EIA-positive samples for AFB_1, STC, and OTA yielded poor quantitative agreement, which was attributed to matrix interference from sample ingredients. This investigation provided evidence that the sample dilutions used in the EIA were insufficient to overcome matrix interference from the constituents present in PBMAs, thus compromising the reliability of the EIA results. As the use of higher dilutions conflicts with sufficiently sensitive detection systems, the use of EIAs for routine mycotoxin analysis in PBMAs requires further investigation to develop a viable sample preparation method.

In conclusion, the studies conducted have shown that EIA is suitable both as a tool for the detection of mycotoxins not found in routine analysis, such as PTA and PAX (**study 1**), and as a rapid screening method to clarify the mycotoxicological quality of PCF products from the German market (**study 2**). However, it was also shown that the EIA reaches its limits for foods of plant origin such as PBMAs due to matrix interferences, which affected the reliability of the EIA results (**study 3**).

7.2 Zusammenfassung

Indolalkaloide wurden bereits in einer Reihe von Lebensmitteln und Getränken in stark variierenden Konzentrationen nachgewiesen. Jedoch ist die Datenlage zum Vorkommen und Nachweis dieser Verbindungen noch stark limitiert. Ein ähnliches Bild der Datenlage ergibt sich für die mykotoxikologische Qualität von speziellen Produkten wie Getreidebeikost für Kleinkinder und Säuglinge sowie für pflanzliche Milchalternativen, die sowohl von Erwachsenen als auch von Kindern konsumiert werden. Die in dieser Arbeit durchgeführten Studien lieferten neue Erkenntnisse zum Nachweis und Vorkommen von Mykotoxinen, insbesondere Indolditerpenen, in der Lebensmittelkette.

Die erste Studie **(Studie 1)** befasste sich mit der Entwicklung eines Enzymimmuntests (EIA) zum Nachweis des Indolditerpenalkaloides Penitrem A (PTA) in methanolischen Myzelextrakten, die aus Pilzkulturen verschimmelter Lebensmittel hergestellt wurden. Um ein möglichst breites Spektrum an Toxinen vom Indoltyp abzudecken, wurden zwei bereits etablierte EIAs zum Nachweis von Paxilline (PAX) und Ergolinalkaloiden in die Versuche einbezogen. Die Analyse der Myzelextrakte mittels EIAs ergab, dass Pilze, die aus verdorbenen Lebensmitteln stammen, PTA, PAX und andere Ergolinalkaloide produzieren. Vergleichsuntersuchungen mittels LC-MS/MS zeigten, dass neben PTA und PAX auch deren Analoga (Penitreme B-F; Desoxy-PAX) in Myzelextrakten detektiert werden konnten. Diese Erkenntnis lieferte Hinweise darauf, dass der PTA-EIA nicht nur PTA, sondern auch dessen Analoga erfasst. Eine weiterführende Untersuchung von ausgewählten PTA-positiven Pilzisolaten mittels Sequenzierung der internen transkribierten Spacer-Region und des β-Tubulin-Gens ergab eine Identifizierung von *P. crustosum* und *P. polonicum*, wobei *P-polonicum* erstmals als PTA-Bildner identifiziert wurde.

Die zweite Studie **(Studie 2)** untersuchte die mikrobiologische und mykotoxikologische Qualität von Getreidebeikost für Kleinkinder und Säuglinge vom deutschen Markt. Untersucht wurde auf eine Reihe mikrobiologischer Parameter (aerobe mesophile Keimzahl, Schimmelpilze, *Enterobacteriaceae*, *Cronobacter* spp., präsumtive *Bacillus cereus*), wobei jeweils mikrobiologische Standardmethoden eingesetzt wurden. In die Mykotoxin-Untersuchungen wurden Deoxynivalenol (DON), Zearalenon (ZEN), Alternariol (AOH), T-2/HT-2 Toxin (nur haferhaltige Proben) sowie Ergotalkaloide einbezogen, wobei die Probenextraktion auf Basis bereits etablierter Methoden erfolgte. **Studie 2** ergab den

Nachweis niedriger Konzentrationen von AOH, DON, T-2/HT-2 (nur haferhaltige Produkte) in Getreidebeikost, wohingegen ZEN und Ergotalkaloide nicht nachgewiesen werden konnten. Ein weiteres wichtiges Ergebnis war, dass einige wenige Proben eine geringe Anzahl opportunistischer Krankheitserreger enthielten, insbesondere *Cronobacter sakazakii*, *Acinetobacter* spp. und *Pantoea* spp. sowie enterotoxigene *Bacillus wiedmannii*. Die innerhalb der Untersuchung erzielten Ergebnisse lagen alle im Rahmen der bestehenden Vorschriften der Europäischen Union oder internationale Richtlinien.

Studie 3 untersuchte die Anwendbarkeit von Mykotoxin-EIAs zur direkten Analyse von pflanzlichen Milchalternativen (PMA) verschiedener Zusammensetzung ohne vorherige Probenextraktion. Zu diesem Zweck wurde zunächst das Ausmaß des Matrixeinflusses von verschiedenen PMA-Matrices auf die EIAs zum Nachweis von Aflatoxin B_1 (AFB_1), Sterigmatocystin (STC), Ochratoxin A (OTA), DON und T-2/HT-2 ermittelt, wobei festgestellt wurde, dass der Matrixeinfluss testspezifisch ausfiel und folglich unterschiedlich hohe Verdünnungen der Proben in den jeweiligen EIAs nötig waren. Nach Analyse der PMA-Proben vom deutschen Markt wurden bei 43 % der Proben positive Ergebnisse in mindestens einem Testsystem erzielt, wobei die meisten positiven Ergebnisse in der Nähe der rechnerischen Nachweisgrenze lagen. Begleitende LC-MS/MS Analysen von EIA-positiven Proben für AFB_1, STC und OTA ergaben nur eine unzureichende quantitative Übereinstimmung, die auf Matrix-interferenzen durch Probeninhaltsstoffe zurückgeführt wurde. Da die Verwendung höherer Verdünnungen im Widerspruch zu ausreichend empfindlichen Nachweissystemen steht, erfordert die Anwendung von EIAs für die routinemäßige Mykotoxinanalyse in PMAs weitere Untersuchungen zur Entwicklung einer praktikablen Probenvorbereitungsmethode.

Schlussfolgernd haben die durchgeführten Studien gezeigt, dass der EIA sowohl als Instrument zum Nachweis von Mykotoxinen, die nicht in der Routineanalytik vorkommen, wie z. B. PTA und PAX (**Studie 1**), als auch als schnelle Screening-Methode zur Klärung der mykotoxikologischen Qualität von PCF Produkten vom deutschen Markt (**Studie 2**) geeignet ist. Es wurde jedoch auch gezeigt, dass der EIA bei Lebensmitteln pflanzlichen Ursprungs wie PMAs aufgrund von Matrixinterferenzen an seine Grenzen stößt, was die Zuverlässigkeit der EIA Ergebnisse beeinträchtigte (**Studie 3**).

7.3 Danksagung

An erster Stelle bedanke ich mich bei meinem Doktorvater Herrn Prof. Dr. Dr. habil. Ewald Usleber für seine ehrliche und immerwährende Unterstützung. Dabei möchte ich mich besonders für die zahlreichen Gespräche auf fachlicher und persönlicher Ebene bedanken, die mir immer als wertvoller Austausch in Erinnerung bleiben werden. Seine fachliche Expertise und vor allem sein leidenschaftliches Interesse für das Forschungsgebiet motivierten mich, mein Ziel stets weiterzuverfolgen. Ich bin ihm sehr dankbar, dass er mir die Möglichkeit zur Anfertigung dieser Arbeit gegeben hat, denn die Faszination von Wissenschaft und Forschung lässt mich heute nicht mehr los und hat so meinen weiteren beruflichen Werdegang entscheidend geprägt.

Frau Prof. Dr. Gesine Lühken danke ich für ihre freundliche Unterstützung als Zweitbetreuerin meiner Arbeit.

Herrn Dr. Benedikt Cramer und Herrn Dr. Ronald Maul danke ich für die Durchführung der Untersuchungen mittels Flüssigkeitschromatographie und die freundliche Kommunikation.

Herrn Dr. Rolf Geisen danke ich recht herzlich für die molekularbiologischen Arbeiten.

Ganz besonders bedanke ich mich bei Herrn Dr. Ömer Akineden für seine großartige Unterstützung, insbesondere im Bereich der Mikrobiologie, sowie für seine verständnisvolle und geduldige Umgangsweise.

Ich danke der Brigitte und Wolfram Gedek-Stiftung für die finanzielle Unterstützung.

Des Weiteren bedanke ich mich ganz herzlich bei allen Mitarbeitern der Professur für Milchwissenschaften, die mich während der Anfertigung meiner Arbeit tatkräftig unterstützt haben. Ich bedanke mich ganz herzlich bei Frau Margit Keßler, Frau Renate Stumpf und Herrn Leon Hart für die vertrauensvolle Zusammenarbeit und die angenehme Arbeitsatmosphäre. Mein Dank gilt ebenso Katrin Seiler und Yann Kusminder für die tatkräftige Unterstützung bei den Arbeiten im Bereich der Mikrobiologie.

Abschließend gilt mein größter Dank meiner Familie, insbesondere meinen Eltern, die immer an mich glauben, mich bedingungslos unterstützen und mir zur Seite stehen.

7.4 Eidesstattliche Erklärung

Erklärung gemäß der Promotionsordnung des Fachbereichs 09 vom 07. Juli 2004 § 17 (2)

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

_____ _____

Ort, Datum Unterschrift